

The development of direct infusion mass spectrometry method for analysis of small metabolites in urine

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*Thesis presented in partial fulfilment of the requirements for the
degree Master of Science in Chemistry
at the University of Stellenbosch*



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March 2013

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Summary

This study focused on the development of an analytical method whereby creatinine, creatine and caffeine could be determined quantitatively. Urine is the preferred body fluid for the analysis of metabolites that the body excretes after administration of medicinal and illicit drugs. The detection of these metabolites depends on the volume of water the patient has drunk or, in criminal cases, the amount of water the suspect may deliberately add to their urine to dilute it. Creatinine, whose concentration in urine has been found to correlate with muscle mass, is chosen as an endogenous control substance against which the metabolite concentration is compared. While high performance liquid chromatography with ultraviolet detection (HPLC–UV) is commonly selected for the analysis, the quality of chromatography is affected by the fact that creatinine, being highly polar, is not retained in the reversed-phase columns. Furthermore, urine contains many polar substances that elute with the solvent front along with creatinine, thereby grossly affecting HPLC measurements. Hydrophilic interaction chromatography (HILIC) is a good alternative, although these methods generally require extensive sample preparation.

Direct infusion electrospray ionization mass spectrometry (DI–ESI–MS) is ideally suited to highly polar compounds and was selected for this work. Pneumatically assisted ESI is preferred above the standard ionization method of atmospheric pressure chemical ionization (APCI) since pneumatically assisted ESI disperses the solution into ion-containing aerosol droplets which do not promote online conversion of creatinine to creatine.

The objective of this study was to develop a simple and sensitive DI–ESI–MS method for the determination of various compounds in urine with creatinine as analytical reference compound and internal standard (IS). The analytical method development includes addition of 1-methyl-3-phenylpropylamine as a primary IS to standard solutions as well as to urine samples, followed by direct infusion of the sample into a mass spectrometer to determine the absolute concentrations of creatinine, creatine and caffeine. After appropriate instrument conditions were established, linear graphs of analyte-IS signal intensity ratios were obtained. The ratio of the concentration of the analyte (drug or metabolite) to that of creatinine (as IS) may be used to determine analyte concentration in artificial samples and/or urine. This method is not affected by change in fluid volume or adulteration of urine samples because the analyte-to-creatinine ratio remains unchanged. As part of this study, the developed DI–ESI–MS method was compared with an LC–UV–MS method developed for this purpose.

Opsomming

Hierdie studie fokus op die ontwikkeling van 'n analitiese metode waardeur kreatinien, kreatien en kaffeïen kwantitatief bepaal kan word. Uriene is die voorkeur liggaamsvloeistof vir die analise van metaboliete wat deur die liggaam, na administrasie van mediese en onwettige middels, uitgeskei word. Die deteksie van hierdie metaboliete hang van die volume water af wat die pasiënt gedrink het, of in strafbare gevalle, die hoeveelheid water wat verdagtes met opset by hul uriene gevoeg het ten einde dit te verdun. Daar is bevind dat die konsentrasie van kreatinien in uriene met spiermassa korreleer, derhalwe is kreatinien as 'n interne kontrolemiddel gekies waarmee die metaboliet-konsentrasie vergelyk kan word. Hoë-druk vloeistofchromatografie met ultravioletdeteksie (HPLC–UV) word algemeen vir die analise van kreatinien ingespan, maar die gehalte van die chromatografie word deur die hoogs polêre aard van kreatinien beïnvloed en het swak retensie in omgekeerde-fasekolomme tot gevolg. Bowendien, uriene bevat groot hoeveelhede polêre middels wat saam met kreatinien in die oplosmiddelfront elueer en sodoende HPLC-bepalings uitermatig beïnvloed. Hidrofiliese interaksiechromatografie (HILIC) is 'n goeie alternatief, ofskoon omvangryke monster-voorbereidings algemeen vereis word.

Direkte inspuitelektrosproei-ionisasie-massaspektrometrie (DI–ESI–MS) is ideaal geskik vir hoogs polêre stowwe en is vir hierdie studie gekies. Pneumatiese hulp-ESI word bo die standaard ionisasie-metode van lugdruk chemiese ionisasie (APCI) verkies weens pneumatiese hulp-ESI se vermoë om die oplosmiddel in aërosoldruppels wat ione bevat, te versprei – sonder die aanlynomskakeling van kreatinien na kreatien.

Die doel van hierdie studie was om 'n eenvoudige en sensitiewe DI–ESI–MS-metode te ontwikkel wat verskeie stowwe in uriene kan bepaal deur kreatinien as analitiese verwysingsmiddel en interne standaard (IS) vir die opstelling van 'n IS-kalibrasiekurwe te gebruik. Die analitiese metode-ontwikkeling sluit die gebruik van 1-metiel-3-fenielpropielamien as primêre IS in. Die IS word tot standaard oplossings en urienemonsters gevoeg, gevolg deur direkte inspuiting van die monster in 'n massaspektrometer om die absolute konsentrasies van kreatinien, kreatien en kaffeïen te bepaal. Lineêre kurwes van die seinintensiteitsverhouding van analiet tot IS is verkry na gepaste instrumentkondisies vasgestel is. Die verhouding van konsentrasie van die analiet (middel of metaboliet) tot dié van kreatinien (as IS) mag gebruik word om die analietkonsentrasie in die standaard oplossings en/of urienemonster te bepaal. Die metode word nie deur veranderinge in die vloeistofvolume of verwatering van urienemonsters beïnvloed nie, weens die analiet-tot-kreatinienverhouding wat onveranderd bly. 'n LC–UV–MS-metode is voorts ontwikkel om die ontwikkelde DI–ESI–MS-metode se data te vergelyk.

Acknowledgements

It is with pleasure and sincere appreciation that I acknowledge each and every one that was part of my journey in writing this thesis.

First and foremost I want to express my gratitude to Dr N. Alpheus Mautjana, my supervisor, who has supported me during my research and preparation of this thesis with his passion, patience, continuous encouragement, stories and words of wisdom.

I also thank my co-supervisor, Dr André J. de Villiers, for his guidance over the last eight months, his willingness to help and for his calm approach when I was faced with some difficulties.

Thank you to Prof Harold Pasch for housing me in one of his laboratories and student offices when I was in need of a place to conduct my research.

With all my love, I specially thank my parents, Gerrit and Tharine, and my siblings, Jannie and Tharine, for their love, praise, encouragement, tremendous support, always believing in my dreams and for all the sacrifices they have made. I am truly blessed to be part of such a loving and caring family.

To all my friends, I want to express my sincere appreciation, in particular René, Henry and Nadia. Their friendship, love and encouragement have been heartfelt and made the journey extraordinary. I want to thank them for sharing this experience with me, for the robust, academic and philosophical conversations, for the mischief we caused, the fun times we had and for being the amazing friends that they are.

Last but not least, I want to thank God for giving me strength and the ability to complete my thesis. This journey would not have been possible without Him.

Table of Contents

Chapter 1: Creatinine – The internal reference compound

1.1	Introduction	1
1.2	Creatinine formation in the body	2
1.3	Role of creatinine in metabolite analyses.....	4
1.4	Separation and detection methods applicable to urinary creatinine	4
1.4.1	Classical Jaffé reaction	5
1.4.2	Electrochemical biosensors.....	5
1.4.3	High performance liquid chromatography.....	5
1.4.3.1	Reversed-phase HPLC.....	6
1.4.3.2	HILIC	7
1.4.4	Tandem mass spectrometry.....	7
1.4.4.1	Direct infusion tandem mass spectrometry.....	7
1.4.4.2	Liquid chromatography tandem mass spectrometry.....	8
1.4.5	Gas chromatography mass spectrometry.....	9
1.4.6	Capillary electrophoresis.....	9
1.4.7	Limitations of current methods for creatinine analysis	11
1.5	Mass spectrometry	11
1.5.1	Electrospray ionization.....	13
1.5.2	Mass analyzer.....	15
1.5.3	Detector	16
1.5.4	Direct infusion electrospray ionization mass spectrometry	16
1.6	Aim of this study	17

Chapter 2: Method development – Quantitative direct infusion mass spectrometry

2.1	Introduction	18
2.2	Experimental procedures	19
2.2.1	Materials and methods	19
2.2.2	Direct infusion mass spectrometry	19
2.2.3	Construction of calibration curves	20
2.2.4	Preparation of unknown and urine sample	21
2.3	Metabolite quantification	21
2.3.1	External calibration method	22
2.3.2	The standard addition method	22
2.3.3	The internal standard method	24
2.4	Proposed method development strategy	25

Chapter 3: Results and discussion

3.1	Preliminary observations	26
3.2	Overcoming sodium adduct ion formation.....	28
3.3	Overcoming creatinine dimerization	28
3.4	The carrier solvent	30
3.5	The primary standard.....	31
3.5.1	2-Phenylbutyric acid.....	31
3.5.2	1-Methyl-3-phenylpropylamine	32
3.6	Calibration curves	33
3.7	Limits of detection and quantification	34
3.8	Quantification of artificial samples	35
3.9	Quantification of urine sample	36

Chapter 4: Method validation – Comparison with HPLC-based quantitation of creatinine

4.1	Introduction	38
4.2	Strategy	40
4.2.1	Development of LC–UV–MS method	41
4.2.2	Metabolite quantification	42
4.3	Materials and experimental procedures.....	43
4.3.1	Chemicals and reagents	43
4.3.2	Materials and instrumentation	43
4.3.3	Procedures for LC–UV–MS method development.....	44
4.3.3.1	Experimental conditions for the reversed-phase column.....	44
4.3.3.2	Experimental conditions for the HILIC column	44
4.3.3.3	Modified methods for the separation of metabolites	45
4.3.4	Procedures for establishing calibration curves	45
4.3.5	Preparation of unknown and urine sample	46
4.4	Results and discussion.....	46
4.4.1	Detection of metabolites by UV.....	46
4.4.2	Separation of metabolites by HPLC.....	46
4.4.2.1	Reversed-phase HPLC method	47
4.4.2.2	HILIC-based separation method.....	47
4.4.2.3	Modified gradient-based separation methods	49
4.4.3	Metabolite quantification	52
4.4.3.1	Calibration curves	52
4.4.3.2	Limits of detection and quantification	52
4.5	Quantification of unknown samples	55
4.6	Comparison of results	56
4.6.1	<i>F</i> -test and Student's <i>t</i> -test	56

4.6.2	Paired t -test.....	59
4.7	Quantification of urine sample	60

Chapter 5: Conclusion

5.1	Overview of achievements.....	63
5.2	Future work.....	64
5.3	Final remarks.....	65

References	66
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List of Abbreviations

ACN	Acetonitrile
AN	Analyte
AP	Atmospheric pressure
APCI	Atmospheric pressure chemical ionization
C _{AN}	Concentration of the analyte
C _{IS}	Concentration of the internal standard
CA	Creatininase
Caff	Caffeine
NH ₄ OOCH	Ammonium formate
NH ₄ COOCH ₃	Ammonium acetate
CI	Creatinase
CNS	Central nervous system
Cnts _{AN}	Absolute counts for the analyte
Cnts _{IS}	Absolute counts for the internal standard
Cr	Creatine
CRM	Charged residue model
Crn	Creatinine
<i>D</i>	Distribution coefficient
DAPPI	Desorption atmospheric pressure photoionization
DART	Direct analysis in real time
dc	Direct current
DESI	Desorption electrospray ionization
DeSSI	Desorption sonic spray ionization
DFG	Deutsche Forschungsgemeinschaft
DI-ESI-MS	Direct infusion electrospray ionization mass spectrometry
ESI	Electrospray ionization
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
H ⁺	Hydrogen ion
HCOOH	Formic acid
HILIC	Hydrophilic interaction chromatography

HPLC	High performance liquid chromatography
IDM	Ion desorption model
IS	Internal standard
LC	Liquid chromatography
LC–MS	Liquid chromatography mass spectrometry
LLS	Linear least squares
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylamide
MALDI	Matrix-assisted laser desorption/ionization
MIP	Molecularly imprinted polymers
MPPA	1-Methyl-3-phenylpropylamine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSD	Mass spectrometer detector
m/z	Mass-to-charge ratio
NaCl	Sodium chloride
NP–LC	Normal-phase liquid chromatography
P	Partition coefficient
PBA	2-Phenylbutyric acid
PCr	Phosphorylcreatine
pK_a	Negative logarithm of the equilibrium constant for an acid in water
Q–TOF	Quadrupole time-of-flight
rf	Radio-frequency
R_t	Retention time
SDS	Sodium dodecyl sulphate
SOx	Sarcosine oxidase
SPE	Solid-phase extraction
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra-violet
VWD	Variable wavelength detector
X^+	Cation
δ -ALA	Delta-aminolevulinate
σ	Residual standard deviation from regression line

Chapter 1:

Creatinine – The internal reference compound

1.1 Introduction

The identification, detection and analysis of the metabolites of new drugs are important for drug discovery, for clinical and forensic toxicology as well as for the identification of illicit drug use. Illicit drugs are a challenge in South Africa and world-wide as these new drugs are developed continuously [1]. It is therefore of great importance to continue developing new methods of analysis and to improve those currently available. Numerous analytical methods are employed to determine the presence of and to quantify illicit drugs in the human body. Typically, body fluid samples such as blood, sweat, saliva and urine are used [2].

Abuse of drugs has been defined as repetitive use of drugs that results in adverse health consequences and social problems. Abuse of drugs often leads to addiction which is a chronic disease of the brain. Addiction has the potential to be fatal if untreated. Addiction cannot be cured, but can be brought into remission through abstinence from all psychoactive substances along with supported recovery [3]. Almost all cases of addiction involve psychoactive drugs which affect the brain and central nervous system (CNS). This class of drugs includes opioids, sedative hypnotics, stimulants, hallucinogens and recently, performance-enhancing drugs such as steroids. Different combinations of drugs may also produce similarly addictive psychological effects. Many drugs like opium, morphine, heroin, ecstasy, lysergic acid diethylamide (LSD), methamphetamine and marijuana have been classified as illegal only in the last half century [2].

The reasons for use and abuse of chemical substances vary. Interestingly, progressive levels of drug use can be easily defined. They begin with abstinence followed by experimentation and then recreational use, drug abuse and finally addiction [3]. Being chemically similar to neurotransmitters that occur naturally in the human brain, psychoactive drugs pass through the blood-brain barrier that protects the brain from foreign materials. Once in the brain they can stimulate or inhibit certain activities, and may block reuptake of the brain's own neurotransmitters [3].

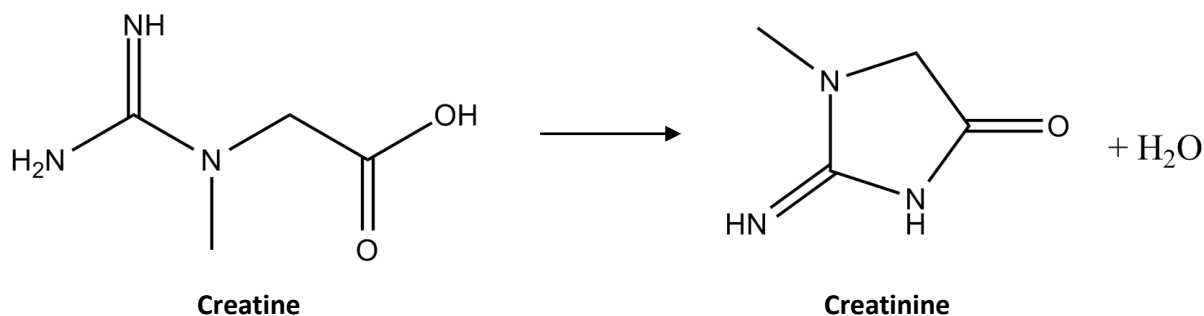
The poor health and social problems resulting from addiction to illicit drugs include cardiovascular complications, impairment of the immune system, neurotoxicity, HIV infection [4] and many other physiological effects such as impaired memory, processing speed and executive functions [5], as well as detrimental, impulsive behavior and decision-making [6, 7].

Different body fluid matrices have been used in the analysis of illicit drugs. The most preferred body fluid to use for the analysis of metabolites in the body following medicinal administration or illicit drug use is urine since taking a urine sample is physically non-invasive. Large volumes of urine can be collected [8, 9]. Compared with other biofluids, urine is simply an aqueous matrix which contains relatively high concentrations of administered drugs and their metabolites [10]. Furthermore, the window for detecting drug abuse with urine often span several days for opiates and cocaine and may be up to months for chronic cannabinoid use while it is limited to only 1–2 days with blood testing. Limitations of testing urine for drugs include adulteration of samples to reduce concentrations of the parent drug that are excreted in urine or its metabolites [9].

The detection of metabolites in urine often depends on the volume of water the patient has drunk or, in criminal cases, on the amount of water deliberately added by the suspect to their urine to dilute it [11, 12]. Adulteration problems can be overcome by using creatinine (Crn), a small polar metabolite whose concentration in urine has been found to correlate with muscle mass [13-16]. Creatinine was used in this work as an endogenous control substance against which concentration levels of metabolite of interest can be compared.

1.2 Creatinine formation in the body

Creatinine, a product of dehydration of creatine (Cr) (Scheme 1.1), was discovered in 1847 by Liebig [17].



Scheme 1.1. Proposed dehydration of creatine to form creatinine.

The exact mechanism of creatine biosynthesis in the body has not yet been fully studied. However, it is largely accepted that the mechanism involves formation of guanidinoacetate in the kidneys, which is transported by blood to the liver where it undergoes methylation to form creatine as shown in Figure 1.1 [17].

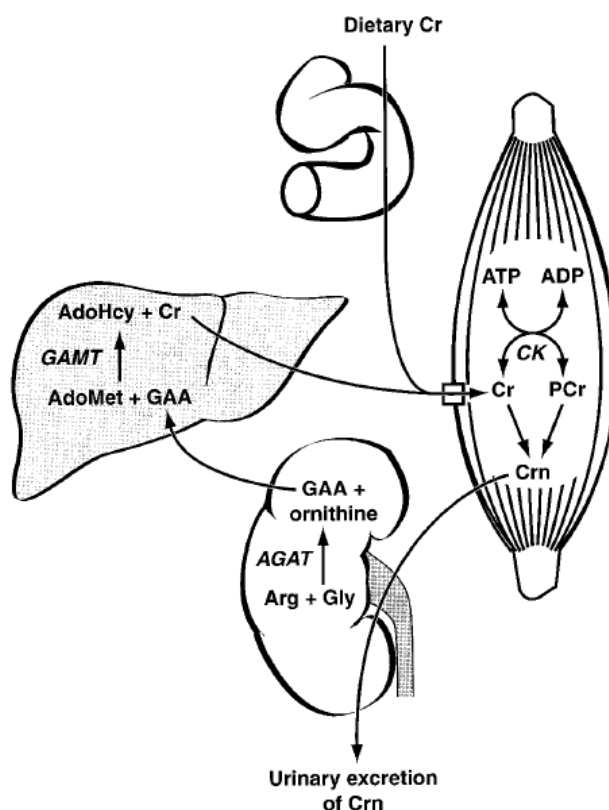


Figure 1.1. Creatine biosynthesis and formation of creatinine [17].

Endogenously generated creatine is exported from the liver by blood and delivered to the organs that require it. Dietary creatine is first absorbed by the intestines and then transported through blood to creatine-requiring tissues [17]. A reversible enzyme-catalyzed phosphorylation of creatine occurs in these tissues to form phosphorylcreatine (PCr). Excess creatine is degraded into creatinine which is subsequently excreted through the kidneys. High levels of creatine and phosphorylcreatine have been detected in skeletal muscles, heart, spermatozoa and photoreceptor cells of the retina [17]. It is important to note that all creatinine is excreted as a waste product [17] and none is reabsorbed or metabolized in the kidneys. This makes creatinine an ideal reference compound for the determination of substance abuse and clinical diagnoses of various diseases [18].

1.3 Role of creatinine in metabolite analyses

Creatinine is usually produced at a fairly constant rate by the body such that its concentration levels correlate with muscle mass. Creatinine is frequently considered to be the best natural internal standard for normalizing the excretion of many metabolites in urine. The concentration of metabolites and diagnostic markers in urine is commonly corrected based on the urinary creatinine concentration [19]. In addition, creatinine is most widely used as a marker of urine dilution and renal dysfunction [20]. Elevated creatine-to-creatinine ratio is used as marker for creatine transporter deficiency [21]. Other metabolites in urine which are used as diagnostic markers for various medical conditions include abnormal concentrations of uric acid and albumin (for hypertension, gouty arthritis, pneumonia, kidney damage, renal death and hyperuricemia) [20, 22], guanidinoacetate as parameter for urea cycle defects [23, 24], pteridines (neopterin, xanthopterin, isoxanthopterin, biopterin) and nucleosides (pseudouridine) as prognostic cancer markers [25-27], and delta-aminolevulinate (δ -ALA) as index of occupational exposure to lead [28].

Recently, a creatinine range of 300 $\mu\text{g/mL}$ – 3000 $\mu\text{g/mL}$ in urine has been adopted as a criterion for specimen acceptance in human biomonitoring studies by the World Health Organization, the American Conference of Governmental Industrial Hygienists as well as by the Human Biomonitoring Commission of the German Federal Environmental Agency, the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) [29]. The normal amount of creatinine in urine, approximately 900–1500 $\mu\text{g/mL}$, is used as an exclusion criterion for deciding on artificially diluted or concentrated samples [26, 29].

1.4 Separation and detection methods applicable to urinary creatinine

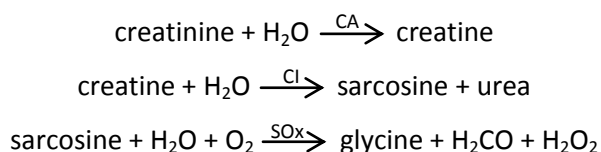
The most recent review of separation methods applicable to urinary creatinine was conducted by Smith-Palmer in 2002 [30]. Since the Smith-Palmer review however, many developments and improvements have been reported. Further updates to the Smith-Palmer review are therefore given here to highlight the general direction of creatinine-based analyses. This section is presented as a background to the new direct infusion mass spectrometry method discussed in the following two chapters.

1.4.1 Classical Jaffé reaction

The Jaffé method [31] is most often employed for the determination of urinary creatinine concentration. Creatinine reacts with picric acid under alkaline conditions and forms an orange-red-colored complex which is detected spectrophotometrically. This reaction is, however, not very specific and prone to interferences by a variety of metabolites in urine, such as glucose, fructose, ketone bodies, ascorbic acid, and cephalosporins [8]. Many studies employ the Jaffé reaction, or colorimetric methods based on the Jaffé reaction, as a comparison to the developed methods for validation purposes [29, 32-36].

1.4.2 Electrochemical biosensors

Enzymatic methods have been developed to enhance specificity in determining creatinine concentration. A three-enzyme method in which creatininase (CA), creatinase (CI), and sarcosine oxidase (SOx) are used to catalyze the hydrolysis of creatinine producing hydrogen peroxide which is detected amperometrically has been reported [37]. The three reactions are shown below.



The catalyzed hydrolysis of creatinine producing ammonia by creatinine iminohydrolase has also been reported, in which the ammonia was detected potentiometrically [38]. The disadvantages of the more creatinine-specific enzymatic methods are the complex immobilization procedures, high cost and the instability of reagents. Mimetic biosensors have also been developed by designing and synthesizing molecularly imprinted polymers (MIP) for creatinine. Sreenivasan and Sivakumar prepared the first MIP for creatinine determination in 1997 [39], after which many investigations into preparation of MIP for creatinine ensued [40-45].

1.4.3 High performance liquid chromatography

The analysis of urinary creatinine has been performed by an array of high performance liquid chromatography (HPLC) methods, including reversed-phase HPLC on a range of different stationary

phases, reversed-phase ion-pair chromatography, and ion-exchange columns as discussed in the review by Smith-Palmer [30]. In this work, the focus is on reversed-phase HPLC and hydrophilic interaction chromatography (HILIC).

1.4.3.1 Reversed-phase HPLC

Reversed-phase HPLC is still used widely for the isolation and quantitation of creatinine in conjunction with various other metabolites, such as methotrexate and several pteridines [26], uric acid [19], tryptophan and tryptophan-related metabolites [46].

Durán Merás *et al.* [26] described the chromatographic separation of creatinine, methotrexate, neopterin, biopterin, pterine-6-carboxylic acid, and isoxanthopterin on a 3.9×150 mm (5 μ m particle size) C18 Nova-Pack column with a 8 min isocratic elution program (1.000 mL/min flow rate) and a mobile phase consisting of 15 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM sodium chloride (NaCl) (pH 6.8). Creatinine was photometrically detected by ultraviolet (UV) absorbance at 230 nm and the rest of the metabolites were detected fluorimetrically with excitation at 280 nm and emission at 444 nm. The two detectors were in series. Creatinine eluted at 1.85 ± 0.01 min with all the metabolites eluting within 7 min. The limit of detection (LOD) was 3.69 μ g/mL for creatinine.

Creatinine and uric acid in human urine were determined by Zuo *et al.* [19] by employing a symmetry C18 reversed-phase column (3×150 mm, 5 μ m) fitted with a 10 mm C18 guard column with a solvent gradient elution program that consisted of sodium phosphate buffer (eluent A), pH 4.75, and acetonitrile (eluent B). The analysis time was 10 min with a flow rate of 0.450 mL/min for the first 3.5 min and increased to 0.500 mL/min for the remainder of the elution. Absorbance was measured at 205 nm. Samples were diluted 100-fold with distilled water and an acid precipitation of protein with phosphorous acid at pH 2.35 was employed before injection. Creatinine (2.970 ± 0.031 min), uric acid and hypoxanthine (internal standard) eluted within 6 min of injection and a LOD of 0.045 μ g/mL for creatinine was reported.

Zhao *et al.* [46] reported the separation of urinary creatinine, tryptophan, kynurenine, kynurenic acid, and 5-hydroxyindole-3-acetic acid. A 4.6×250 mm (5 μ m particle size) Agilent HC-C18 column was used and a 30 min gradient elution program at a constant flow rate of 1.000 mL/min and ambient temperature. The mobile phase consisted of eluent A (20 mM sodium acetate, 30 mM acetic acid and 3% methanol) and eluent B (20 mM sodium acetate/acetic acid, 10% methanol and

10% acetonitrile). Creatinine, kynurenine and kynurenic acid were measured with a variable wavelength detector (VWD) at 258 nm, 365 nm (at 9 min) and 344 nm (at 14 min), respectively. Tryptophan and 5-hydroxyindole-3-acetic acid were fluorimetrically determined with excitation at 295 nm and emission at 340 nm. Urine samples were diluted five-fold before injection. The LOD for creatinine was 0.2 µg/mL with a retention time (R_t) of 4.18±0.00 min.

All three methods have time-consuming sample preparation procedures, although the HPLC analysis in the first two methods for the simultaneous determination of creatinine and the other metabolites are fast. Analysis of the last method is complicated by the characteristics of the compounds. Zuo *et al.* [19] reported the lowest LOD for creatinine.

1.4.3.2 HILIC

A HILIC method was developed for simultaneous determination of urinary creatinine and uric acid by Zuo *et al.* [20] with cimetidine as internal standard. Urine samples were diluted 100-fold after which protein precipitation, centrifugation and filtration were carried out. Isocratic elution was used for separation on a S₅NH₂ column (4.6 × 250 mm, 5 µm) with an NH₂ guard column (4.6 × 7.5 mm, 5 µm) within 6 min at a flow rate of 1.200 mL/min and UV detection measured at 205 nm. The mobile phase consisted of 50% acetonitrile and 50% 10 mM sodium phosphate buffer solution (pH 4.75). Creatinine eluted at 3.08±0.01 min. The LOD was 0.04 µg/mL for creatinine and 0.06 µg/mL for uric acid. This method proved to be fast, accurate and reliable as well as being simple and robust when compared with reversed-phase HPLC methods.

1.4.4 Tandem mass spectrometry

1.4.4.1 Direct infusion tandem mass spectrometry

Huškova *et al.* [36] used isotope dilutions for the analysis of urinary creatinine with a d₃-labeled isomer as the internal standard. Two methods were evaluated, one analyzing samples without pretreatment (WP-TMS method) and one after solid-phase extraction (SPE) cation-exchange clean-up (SPE-TMS method). The LOD for creatinine was 0.2 µmol/L (22.6 ng/mL) for both methods.

1.4.4.2 Liquid chromatography tandem mass spectrometry

Park *et al.* [47] performed a liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis on a XTerra MS C18 column (2.1 × 30 mm, 3.5 µm) and an eluent consisting of 50% acetonitrile and 0.1% formic acid with a flow rate of 0.300 mL/min at ambient temperature. The ion transitions at mass-to-charge (m/z) ratios of 114.0 → 44.0, were monitored in multiple reaction monitoring (MRM) mode. This method had a LOD of 1 ng/mL for creatinine.

Isotope dilution electrospray tandem mass spectrometry, using d_3 -labeled creatinine as the internal standard, has been used for the simultaneous separation of urinary creatinine and uric acid [35]. A multi-mode ODS column (2 × 75 mm, 3 µm) fitted with a guard column (2 × 5 mm, 3 µm) was used with the mobile phase consisting of 0.2% formic acid (eluent A) and acetonitrile (eluent B) at a flow rate of 0.150 mL/min. The total analysis time was 11 min with creatinine eluting at 1.20 min. The HPLC system was coupled to a QTrap triple-quadrupole mass spectrometer with electrospray ionization (ESI) operated in positive mode for creatinine and negative mode for uric acid analysis. Detection was carried out in MRM mode using the ion transitions (114.0 → 86.0 and 114.0 → 44.0 m/z) and (166.9 → 124.1 and 166.9 → 95.9 m/z) for creatinine and uric acid, respectively. The sample preparation involved centrifugation of the urine samples to obtain clear supernatants at 50 000 g for 3 min. The samples were diluted 40-fold with distilled water after which it was diluted again three-fold with the internal standard solution and acetonitrile. The solution was filtered before injection for LC–MS/MS analysis. A LOD of 30 ng/mL for creatinine was obtained.

A HILIC method by Goucher *et al.* [48] involved the simultaneous extraction, separation and detection of creatinine and the opioid methadone, as well as methadone's primary metabolites (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline) in human urine. SPE was performed prior to HPLC analysis and the total runtime was 3 min. An amide-80 (4.1 × 250 mm, 3 µm) HILIC carbamoyl phase column with an isocratic mobile phase containing 28% (v/v) acetonitrile (with 0.01% formic acid) and 72% 3 mM ammonium formate in water (with 0.01% formic acid) and a flow rate of 0.250 mL/min was used. MS/MS analysis was performed in positive ESI and MRM mode. The ion transition 114.3 → 44.6 m/z was used for quantification. The R_t was 1.81±0.6 min for creatinine with a LOD of 0.250 ng/mL.

Very low LODs are obtained with MS/MS analyses compared with HPLC. This is a clear indication of the sensitivity of MS detectors.

1.4.5 Gas chromatography mass spectrometry

The analysis of creatinine by gas chromatography (GC) requires the derivatization of the compound to facilitate their movement through the column. Isotope dilution gas chromatography mass spectrometry (GC–MS) methods have been established for the analysis of urinary creatinine alone [29] and in conjunction with guanidinoacetate [23] and creatine [49].

Creatinine was derivatized with bis(trimethylsilyl) tri-fluoroacetamide and the ion was measured at 258 m/z in the method employed by Arias *et al.* [23]. MacNeil *et al.* [49] determined the concentrations of creatinine and creatine by HPLC and performed separation of the two compounds with cation-exchange chromatography after which each fraction was derivatized with trifluoroacetic anhydride to determine the ratios of creatinine:creatinine- d_3 and creatine:creatinine- d_3 by GC–MS analysis. A new GC–MS method was reported by Tsikas *et al.* [29] for the quantitative determination of creatinine in human urine. The derivatization reagent 2,3,4,5,6-pentafluorobenzyl bromide was used to derivatize creatinine and selected-ion monitoring of 112 m/z was performed. The LOD was reported to be 100 amol.

1.4.6 Capillary electrophoresis

Analysis of creatinine by capillary electrophoresis (CE) and microchip capillary electrophoresis (μ CE chip) has enjoyed considerable attention over the past 10 years. The advantages attributed to CE are high separation efficiency, fast analysis speed, multiple separation modes, and excellent biocompatibility. The analysis of complex matrices such as urine requires pretreatment procedures to suppress interferences. These procedures are tedious and time-consuming [50]. Simultaneous analysis of creatinine and other compounds such as creatine, guanidinoacetic acid, uric acid, *p*-aminohippuric acid, serotonin, and nucleosides in human urine have been performed.

Costa *et al.* [51] analyzed creatinine in urine (20-fold dilution) in 22 s, using a buffer composed of 10 mM Tris and 20 mM 2-hydroxyisobutyric acid at pH 3.93. Separation was performed on a fused-silica capillary (8.5 cm effective length), with direct UV detection at 215 nm. Simultaneous separation and detection of creatinine, creatine and guanidinoacetic acid was reported by Zinellu *et al.* [52] using an uncoated fused-silica capillary (50 cm effective length) and a 75 mM Tris buffer (pH 2.25) at 15°C with a 30 kV applied voltage. The separation was completed within 8 min with detection of the

compounds by a spectrophotometer. Urine samples were diluted 20-fold with water before injection.

Szymańska *et al.* [34] applied an extensive SPE procedure to undiluted urine samples in order to separate 13 nucleosides. Separation was conducted in a fused-silica capillary (70 cm effective length) with a background solution containing 100 mM borate, 72 mM phosphate and 160 mM sodium dodecyl sulphate (SDS), pH 6.7, at 30°C with a 25 kV separation voltage applied to the capillary. The nucleosides and creatinine were detected spectrophotometrically. The analysis was completed after 26 min. Creatinine was separately analyzed under the same conditions with a migration time of 10 min. Jiang and Ma [53] reported a dramatically reduced separation time of 7.5 min for ten modified nucleosides in urine samples. Creatinine was also analyzed separately. Electrophoretic separation was achieved in a fused-silica capillary (38 cm effective length) at –15 kV applied voltage and 25°C with a buffer, which contained 25 mM borate, 25 mM phosphate and 25 mM cetyltrimethylammonium bromide at pH 9.5.

A serial ultrasound-assisted emulsification microextraction procedure for urine pretreatment was employed by Huang *et al.* [50] to analyze creatinine and serotonin. The separation was concluded after 15 min migration time on a 48 cm effective length fused-silica capillary and 30 mM Tris-phosphate buffer, at pH 2.85, with an applied voltage of 20 kV. The method provided a sensitivity enhancement of 360-fold for the detection of serotonin.

Lee and Chen [54] reported the separation of creatinine and uric acid in human urine in 400 s by incorporating a multiple-enzyme (CA, CI and SOx) assay into a μ CE chip with electrochemical detection. Urine samples were diluted ten-fold and filtered before injection. Wang *et al.* [55] used the same enzymatic assay and electrophoretically separated creatinine, creatine, uric acid, and *p*-aminohippuric acid and amperometrically detected reaction products. Application of this method to a 50-fold diluted urine sample yielded a separation and detection of creatinine and uric acid within 400 s, however *p*-aminohippuric acid was not detected. Garcia *et al.* [56] described the separation of creatinine, creatine and uric acid in a urine sample (20-fold dilution) within 150 s by pulsed electrochemical detection using a buffer consisting of 30 mM borate (pH 9.4) and 1 mM SDS.

1.4.7 Limitations of current methods for creatinine analysis

The limitations of the current methods for analysis of creatinine are summarized in Table 1.1. None of these methods directly address the problem of sample adulteration and most require cumbersome and time-consuming sample preparation and analysis procedures.

Table 1.1. Limitations of current methods for analysis of creatinine.

Method	Limitation(s)
Classical Jaffé reaction	Non-specific for creatinine
Electrochemical biosensors	Complex immobilization procedures, high cost and instability of reagents
Reversed-phase HPLC	Cumbersome and time-consuming sample preparation and analysis procedures
HILIC	High cost
Direct infusion MS/MS	-
LC-MS/MS	High cost and time-consuming sample preparation and analysis procedures
GC-MS	High cost and time-consuming sample preparation procedures (derivitization)
CE	Time-consuming sample preparation and analysis procedures

1.5 Mass spectrometry

The birth of MS is attributed to Sir Joseph J. Thomson who discovered the electron in 1897 and he constructed the first mass spectrometer in 1902 [57].

MS has unique capabilities which have made it widely popular. Firstly, it provides unsurpassed molecular specificity because of its unique ability to measure accurate molecular mass. Secondly, it provides ultrahigh detection sensitivity. Thirdly, it has unparalleled versatility to determine the structures of most classes of compounds and individual elements. Furthermore, it is applicable to a large variety of samples, volatile and non-volatile, polar or non-polar. Lastly, it can be applied in combination with high resolution separation devices [58].

All mass spectrometers consist of five essential components, namely an inlet, ion source, mass analyzer, detector, and data system (Figure 1.2) [58]. It can be arranged in many instrumental configurations, each with its specific advantages, limitations and cost-to-benefit ratio. Novel ionization methods, mass analyzers and sample pretreatment techniques are continually being developed, improved and utilized.



Figure 1.2. Essential components of a mass spectrometer.

MS has become one of the most relevant techniques in drug development, profiling of metabolomes, clinical and forensic toxicology, analyses of polymers [59] and many more. Due to its high sensitivity and specificity, MS hyphenation with chromatographic procedures has proved to be invaluable in the analysis of drugs, toxins, and/or their metabolites in complex biological samples such as blood or urine, or alternative matrices such as hair, saliva, sweat, or meconium [60, 61]. Both LC–MS and GC–MS have become particularly relevant in the detection of illicit drugs [62]. In hyphenated cases the MS instrument is used as a detector. In the case of LC–MS the effluent from the HPLC column enters the ionization chamber through the ESI needle as shown in Figure 1.3.

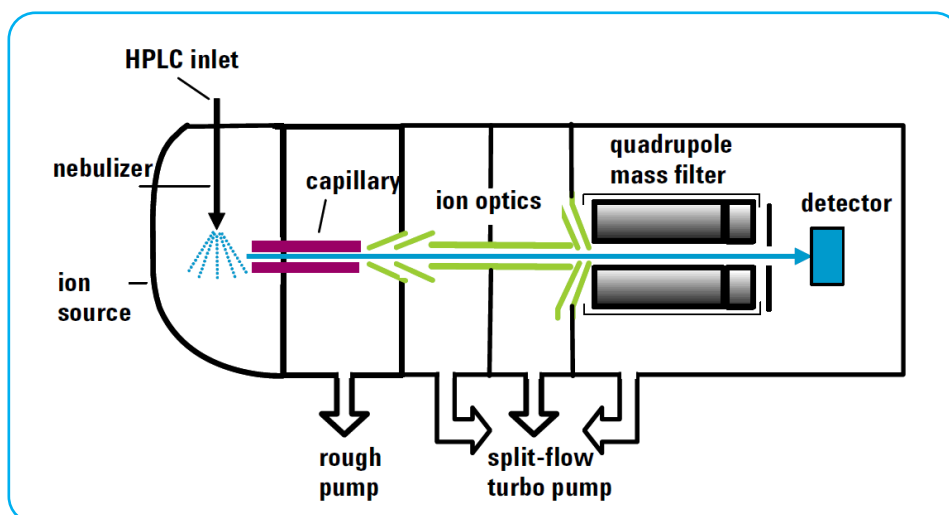


Figure 1.3. Block diagram for an Agilent quadrupole MS system.

The LC–MS set-up allows the identification of the compounds of interest so that they can be quantitatively analyzed by the chromatographic technique. However, many analytes require pretreatment of the sample in order to make them volatile in the case of GC–MS or to make them responsive to light in the case of LC–UV. It should be noted that sample treatment is time consuming.

In recent years, advances have been made to conventional MS interfaces such as matrix-assisted laser desorption/ionization (MALDI) under vacuum [63, 64] and ESI techniques to allow more direct analysis of samples by mass spectrometry. These techniques include atmospheric pressure MALDI [65], desorption electrospray ionization (DESI) [66, 67], desorption atmospheric pressure photoionization (DAPPI) [68], direct analysis in real time (DART) [69, 70] and desorption sonic spray ionization (DeSSI) [71] among others. These approaches still fall short with regard to quantitative determination.

1.5.1 Electrospray ionization

ESI is based on the application of high voltage to sample flow to produce small droplets. The discovery of electrospray phenomenon can be dated back to 1750, when Jean-Antoine Nollet observed that water flowing from a small hole of an electrified metal container forms aerosol when placed near the electrical ground [72]. Later, a series of systematic studies on electrospray were carried out by Zeleny [73-75] and Taylor [76] allowing a detailed description of the phenomenon. Electrospray was mainly put to use as an effective painting technique, but in the 1960s and early 70s Dole *et al.* reported the first use of electrospray as an ion source for mass spectrometry when they produced gas-phase, high molecular weight polystyrene ions by electrospraying a benzene/acetone solution of the polymer [77, 78]. The latest basic developments of ESI–MS were made by Fenn *et al.* [79-81] for which John B. Fenn received the Nobel Prize in Chemistry in 2002 [82].

The mechanism of ESI can be described in three main steps. The first step involves the formation of charged droplets at the tip of the spray capillary. In conventional ESI, the charging of the droplets is due to the action of the applied electric field between the spray capillary tip and a counter electrode. A charge separation takes place and an enrichment of ions of the same polarity as that of the emitter will occur at the emitter. In pneumatically assisted ESI, co-axial flow of heated nitrogen gas is introduced to enhance droplet formation and ionization in the ion source with the spray capillary grounded. An electric field is applied between an electrode in the spray chamber and the

inlet capillary which serves as the counter electrode. If the field is strong enough, charged droplets are directed to the heated capillary inlet and naked ions are formed as the solvent evaporates. This ionization phenomenon is strongly enhanced by the presence of hydrogen ions. The eruption of charged droplets into naked ions is strongly influenced by the solvent physico-chemical properties (viscosity, surface tension, pK_a), the concentration and chemical nature of analytes as well as ionizing agents i.e. acid, and the voltage applied between inlet capillary and counter electrode [58, 83, 84].

The second step entails the evaporation of solvent from the droplets. When a charged droplet travels towards the counter electrode, its radius decreases as the solvent evaporates, but its charge remains constant. Solvent evaporation is achieved by the flow of hot nitrogen, which also heats up the spray chamber. Evaporation of the charged droplets is further enhanced by a heated capillary. The decrease in droplet radius leads to an increase in surface charge density. At a certain point, called the Rayleigh limit, the Coulombic force overcomes the surface tension of the liquid and the droplet undergoes irregular fission into several offspring droplets. This so-called Coulombic fission can be repeated in several cycles, leading to very small highly charged second-generation droplets [58, 83, 84].

The final step is the formation of gas phase ions. The actual mechanism for the transfer from solvated ions to gas phase ions is not fully understood and has been under discussion for a long time. Two main theories have been suggested: the charged residue model (CRM) and the ion desorption model (IDM). Lately it has been suggested that both mechanisms apply. The CRM involves a process of repeated sequential solvent evaporation and a series of scissions which lead to the production of small droplets containing only one solute molecule. As the last solvent molecules on each droplet evaporate, analyte molecules are dispersed into the ambient gas, retaining the charge of the droplets. The IDM also relies on the sequence of solvent evaporation and fission of the droplets. This model, however, proposes the expulsion of the solvated ions into the gas phase at some intermediate droplet size when the electric field due to the surface charge density is sufficiently high but less than the Rayleigh instability limit [58, 81, 83, 84].

The ESI source can lead to the production of positive or negative ions, depending on the polarity of voltage applied to the sprayer and the counter electrode [84] or in the case of pneumatically assisted ESI with the spray capillary grounded, between an electrode in the spray chamber and the inlet capillary. ESI is by far the method of choice in LC-MS metabolomic studies because it produces large numbers of ions via charge exchange in solution. The unique formation of molecular ion

species in the gas phase makes the ESI method highly interesting for the analysis of complex mixtures. If these ions were transferred reproducibly, quantitative analysis by MS would be carried out directly without the need of prior chromatographic separation. Through a simple direct infusion of the mixture dissolved in a suitable solvent it is possible to obtain relative ratios of the molecular species present in the mixture itself.

1.5.2 Mass analyzer

The mass analyzer is the heart of a mass spectrometer. It is a fundamental part which separates and mass-analyzes the ionic species according to their mass-to-charge (m/z) ratio and focuses all mass-resolved ions at a single focal point. The m/z is by definition the mass of an ion (m) divided by the number of charges (z) the ion carries. The motion of the ions is controlled by magnetic and/or electric fields. The most common forms of mass analyzers include a quadrupole, time-of-flight, magnetic sector, orbitrap, quadrupole ion trap, and Fourier transform ion cyclotron resonance instrument. A mass analyzer is best defined according to its ion transmission efficiency and mass resolution. Ion transmission efficiency involves the ability of a mass analyzer to deliver various ions in the entire mass range to the detector. This is also a reflection of the sensitivity of the instrument. By definition, mass resolution is the analyzer's capability to distinguish between two neighboring signals of ions that differ only slightly in their mass (Δm) [58, 83].

The quadrupole mass analyzer is shown in Figure 1.4. It consists of four cylindrical rods that are parallel to one another. Direct current (dc) and radio-frequency (rf) potentials are applied to these symmetrically arranged rods. The field within the square array is created by alternating voltages between opposite pairs of electrodes. The ions accelerated along the z -axis enter the space between the rods and maintain their velocity along this axis. Due to the electric field, these ions are also accelerated in the x and y -directions. Ions with a specific m/z value pass through the geometry of the rods when a set of defined dc and rf potentials are applied because of their stable trajectories. In the xz -plane, a positive ion will be accelerated toward the central axis when a positive dc potential is applied to the pair of rods in the same plane. The simultaneous action of the rapidly changing rf potential during its negative half-cycle will accelerate these ions towards the rods. The positive electrodes act as a high-pass filter. The pair of electrodes in the yz -plane is at a negative polarity. The positive ions will be attracted toward these rods and during the positive half-cycle of the rf potential only the ions with lower m/z will be focused to the central axis. The negative electrodes act as a low-

pass filter. The combination of these actions creates a stability window for ions of a narrow m/z range to travel through the rods in the z -direction [58, 83].

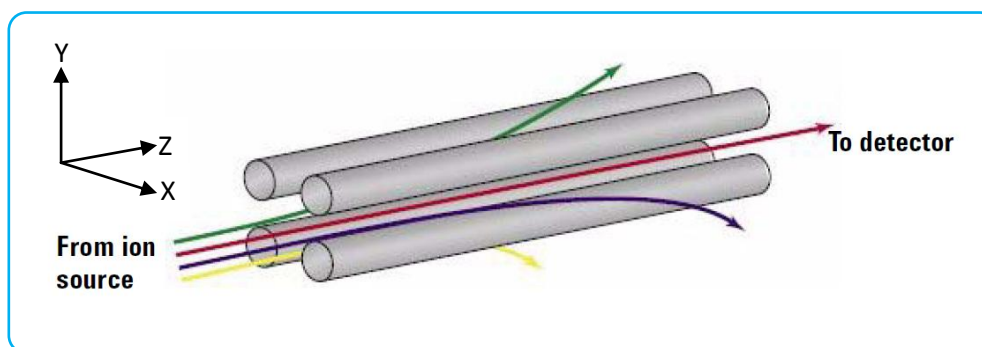


Figure 1.4. Quadrupole mass analyzer.

The low cost, mechanical simplicity, high scan speeds, high transmission, increased sensitivity, independence from the initial energy distribution of ions, and linear mass range are advantageous attributes of a quadrupole mass analyzer [58].

1.5.3 Detector

The detector is responsible for converting the ion current into signals. The MS used in this work was equipped with the electron multiplier detector. The ions from the mass analyzer are accelerated to a high velocity in order to enhance detection efficiency by holding the conversion dynode at a high potential (from ± 3 to ± 30 kV) opposite to the charge polarity of the detected ions. A positive ion striking the conversion dynode causes the emission of several electrons. These are then amplified by a cascade effect to produce an amplified electric signal [83].

1.5.4 Direct infusion electrospray ionization mass spectrometry

Direct infusion electrospray ionization mass spectrometry (DI-ESI-MS) is ideally suited to highly polar compounds and was selected for this work. Its advantages include high sensitivity, high selectivity, wide dynamic range, robustness, and the ability to identify metabolites [85]. In contrast to LC, the use of DI-ESI-MS has the greatest potential for high peak capacity and sample throughput with minimal or no sample pretreatment. Complex samples can be analyzed without sample separation steps [86, 87]. The application of DI-ESI-MS to highly complex and variable samples such as urine and plasma/serum where matrix effects are inevitable has a number of challenges. Such

challenges include ion suppression or undesired signal enhancement, and difficulties in separating isobaric substances [87, 88]. DI-ESI-MS has been applied in various fields of research, including the study of blood plasma metabolites [89], urinary metabolites [90], human metabonomics and metabolomics [86-88], fungi and yeast metabolomics [91, 92], secondary metabolites from microorganisms [93], fruit and vegetable metabolomics [94-98], food [99, 100], polymers [101], and characterization of proteinaceous glues [102].

1.6 Aim of this study

The objective of this study was to develop a simple and sensitive DI-ESI-MS method for the determination of various compounds in urine with creatinine as analytical reference compound and internal standard (IS) for the construction of an IS calibration curve. When fully developed, this method will not be affected by change in fluid volume or adulteration of urine samples because the analyte-to-creatinine or analyte-to-creatine ratio remains unchanged.

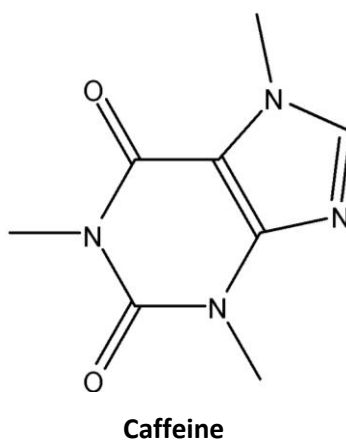
Chapter 2:

Method development – Quantitative direct infusion electrospray mass spectrometry

2.1 Introduction

Creatinine and creatine are small, highly polar metabolites found in urine. Although these compounds have been analyzed with liquid chromatography (LC) and gas chromatography (GC)-based methods, they are associated with some limitations. In reversed-phase LC, these compounds elute with the solvent front while in normal phase, they tend to stick to the stationary phase. Since these metabolites are highly polar and typically have very high boiling points, they are unsuitable for analysis by GC unless they are chemically derivatized. Other different analytical methods for metabolites in urine are limited in that they require extensive sample pretreatment and are in many cases quite cumbersome and tedious as evident in the review of Smith-Palmer [30] and the discussion in section 1.4.

In this work, a simple and sensitive direct infusion electrospray ionization mass spectrometry (DI-ESI-MS) method was developed for the detection and quantification of creatinine, creatine (Scheme 1.1) and caffeine (Scheme 2.1). Caffeine was selected as a model compound to represent common drugs or drug metabolites that may be analyzed.



Scheme 2.1. Chemical structure for caffeine.

2.2 Experimental procedures

2.2.1 Materials and methods

All chemical and chromatographic reagents used were of HPLC grade. Creatinine, creatine, caffeine, 2-phenylbutyric acid, 1-methyl-3-phenylpropylamine (MPPA), methanol and acetic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and were of the highest purity. Millipore water (Millipore, Milford, Massachusetts, USA) was used in all solutions and analyses. Mass spectrometry (MS) was performed on an Agilent 6120 LC/MSD Single Quad purchased from Agilent Technologies (Böblingen, Germany) connected to a N2-Mistral-LCMS nitrogen generator purchased from LNI Schmidlin SA (Geneva, Switzerland). The system consists of two regions: (A) Ion source and (B) Ion transport and focusing region. The atmospheric pressure ionization source is an ESI source. The ion transport and focusing region consists of four stages which are under vacuum: (A) Inlet capillary and fragmentation zone; (B) Skimmers; (C) Octopole; (D) Quadrupole mass analyzer and electron multiplier detector. All statistical and graphical analyses were performed with SigmaPlot® 11.0 (Systat Software, Inc., Chicago, Illinois, USA).

2.2.2 Direct infusion mass spectrometry

The determination of concentration of creatinine, creatine and caffeine by ESI-MS involves continuous infusion of the analyte solution. The analyte solution enters the needle by gravitational force alone, since a pump is not used, ensuring a consistent and reproducible flow speed. The MS conditions for the direct infusion analysis are summarized in Table 2.1.

Table 2.1. MS conditions for direct infusion analysis.

Mass analyzer mode	Scan
Ion polarity mode	Positive
Mass-to-charge range	20–300 m/z
Capillary voltage	-4000 V
Chamber voltage	-3500 V
Fragmentation voltage	95 V
Scan time	5 min

A schematic diagram of the Agilent 6120 MS instrument, which was used for the direct infusion experiments, is shown in Figure 2.1.

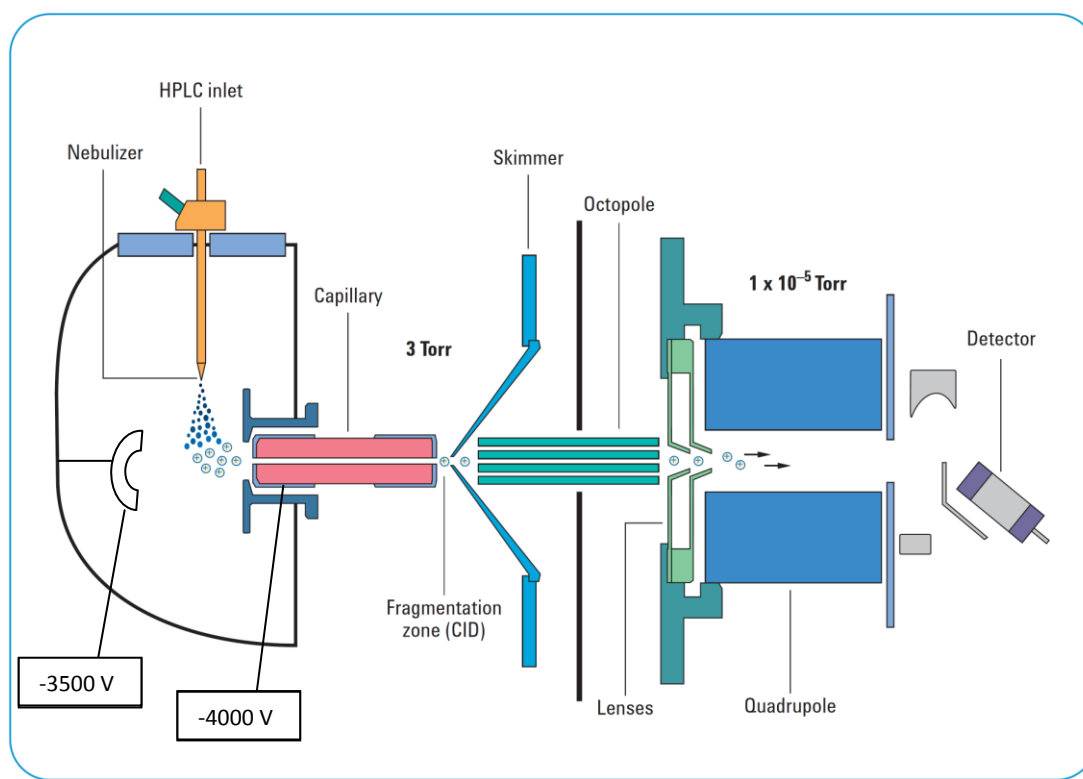


Figure 2.1. Agilent 6120 Mass Spectrometer.

The analyte solution is continuously introduced by the ESI needle to the ion source in the spray chamber which is a high voltage region. The needle is placed inside a larger capillary through which nitrogen gas is pumped to nebulize the solution. Upon nebulization the analyte molecules are ionized by cations (X^+), predominantly H^+ ions from solution, forming $[M+nX]^{n+}$ ions. The charged droplets pass across an electric field between the electrode at a higher voltage (-3500 V) relative to the heated capillary held at -4000 V. The ions are electrostatically guided and drift towards the relatively high negative voltage; in doing so, the ions enter the heated capillary and proceed into the analyzer region. Data processing involved multiple scanning of the mass range (20–300 m/z) ions for about 5 min to obtain an average mass spectrum with averaged signals for each ion detected.

2.2.3 Construction of calibration curves

Stock solutions containing 1 mg/mL standard were prepared for creatinine, caffeine and MPPA in distilled water and stored in a cool, dry place. Dilutions of each standard stock were made with a solvent containing 90:7.5:2.5 (% v/v/v) methanol, water and acetic acid. Two sets of calibration

curves were constructed. The first set contained creatinine as the analyte and MPPA as primary internal standard. The standard mixtures were made in triplicate to contain 1, 2.5, 5, 7.5 and 10 µg/mL of creatinine, respectively, and each with 5 µg/mL of MPPA, the primary internal standard. The second set contained caffeine as the analyte and creatinine as secondary internal standard. The standard mixtures were made in triplicate to contain 1, 2.5, 5, 7.5 and 10 µg/mL of caffeine, respectively, and each with 5 µg/mL of creatinine, the secondary internal standard.

Triplicate measurements of each standard were made by DI-ESI-MS. Blank runs were performed after every measurement to ensure that there was no carry-over between runs. The ratio of the absolute counts (abundance) of the m/z signals and the concentration ratio of the analyte (AN) and internal standard (IS) were calculated: $(\text{Cnts}_{\text{AN}})/(\text{Cnts}_{\text{IS}})$ and $C_{\text{AN}}/C_{\text{IS}}$ respectively. Calibration curves of $(\text{Cnts}_{\text{AN}})/(\text{Cnts}_{\text{IS}})$ versus $C_{\text{AN}}/C_{\text{IS}}$ were constructed. The calibration curves displayed a linear relationship over the concentration range of 1–10 µg/mL for the analyte and were fitted with a linear equation (given below) by linear regression in SigmaPlot® 11.0.

$$y = mx + c \quad \text{Eq. 1}$$

2.2.4 Preparation of unknown and urine sample

The three samples with unknown amounts of creatinine and caffeine were prepared by a laboratory at the Department of Biochemistry, Stellenbosch University (Stellenbosch, South Africa). Urine from a subject was collected over 24 hours. After each collection, the urine was immediately stored at 4°C. After 24 hours the collections were pooled and a sample was taken. Before analysis, the unknown and urine samples were kept at room temperature for 30 min. 0.5 mL of a 100 µg/mL stock solution of the internal standard, MPPA, in distilled water was added to 100 µL of unknown or urine sample in a 10 mL volumetric flask. Solvent (90:7.5:2.5 (% v/v/v) methanol, water and acetic acid) was added to the mark to obtain a 100-fold dilution. The samples were then ready for DI-ESI-MS analysis.

2.3 Metabolite quantification

The quantification of urinary metabolites with MS is usually performed by coupling the mass spectrometer to another analytical instrument where the mass spectrometer serves as a

complementary technique. In this work quantification with DI–ESI–MS was assessed. Three common methods of quantification, namely, external calibration method, standard addition method and the internal standard method were considered as discussed below.

2.3.1 External calibration method

External calibration is the most common method determining analyte concentration in a sample. The method involves construction of a calibration plot of the instrument response versus concentration of standard solutions. The response of an unknown is used to read its concentration from the calibration graph. Comparison of the instrument response of a sample directly to that of a single standard solution is unreliable. It is therefore of utmost importance that multiple standards are used to ensure greater reliability of the results. Analyte concentration in a given sample is determined either visually from the graph as a value corresponding to the sample signal or by calculation using the equation of the graph [103].

The external calibration method has several desirable characteristics and is widely used in quantitative instrumental analysis. The two most common advantages of external calibration are its general applicability and efficiency in routine analyses. Once the calibration graph has been prepared, a large number of samples which may vary can be analyzed. However, frequent confirmation of readings of the standards is necessary to ensure that the instrument response is constant [103]. The greatest disadvantage of external calibration arises from the fundamental assumption that the instrument response observed for the standards remains unchanged whenever the unknown is measured. Most unknown samples contain a matrix, components not present in the standards, which often affect the response. Such matrix components do often give rise to systematic error in the results, unless their effect is corrected or they are removed [103].

2.3.2 The standard addition method

Some analyses are only possible with standard additions. In general, the standard addition method is used to eliminate the effects of the sample matrix in the results. The method is carried out by first dividing the sample which contains the analyte of interest into several solutions to which a known amount of a pure standard is added. The amount of analyte in the unknown (U) sample remains constant while the amount of standard (S) added increases proportionally with each increment. The equation of the graph (Figure 2.2) is used to determine the intercept, V_S' —which corresponds to the

negative of the amount of analyte in the original unknown—once the line has been extrapolated to the x-value that corresponds to the y-value of zero [103]. The unknown concentration of the analyte in the original sample mixture is determined by calculation using equation 2,

$$C_U = \frac{-C_S \times V_S'}{V_U} \quad \text{Eq. 2}$$

where C_U is the concentration of the unknown, C_S is the concentration of the standard, V_S' is the intercept of the x-axis (volume of standard) and V_U is the volume of the unknown.

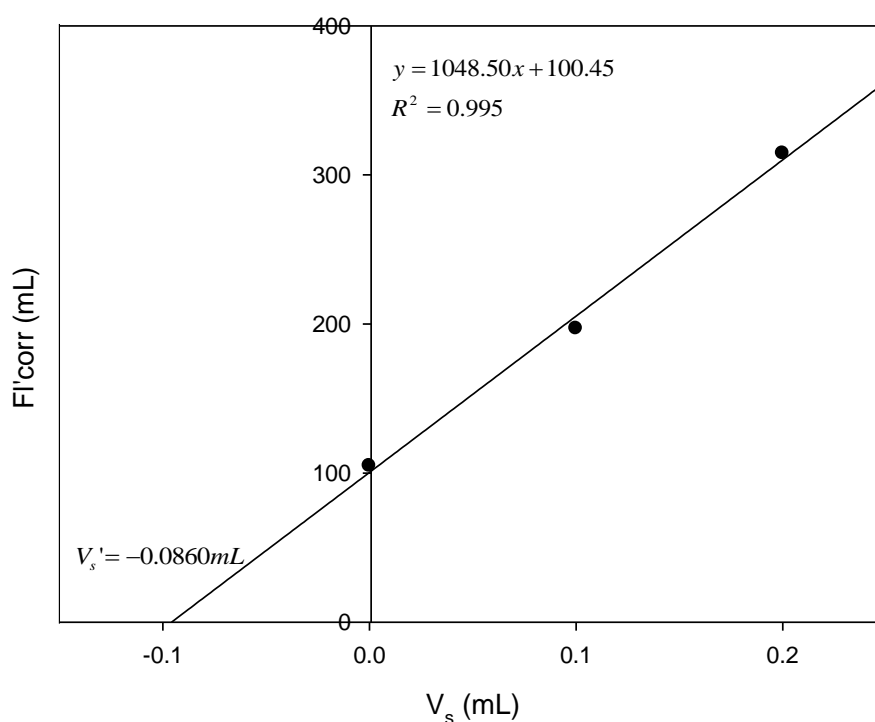


Figure 2.2. Typical standard additions plot.

While it allows reduction of the matrix effects on the response of the instrument and thereby increasing reliability of the results, the standard addition method is suitable only for non-routine analyses. Its calibration plot is good for a given, single sample or set of very similar samples. Further limitations include additive matrix effects. The standard addition method is affected greatly by fluctuations in instrument response for a given sample and is therefore not suitable for quantitative analysis by DI-ESI-MS [103].

2.3.3 The internal standard method

The internal standard method, otherwise known as internal standardization, is ideal when variations in sample sizes and instrument response are encountered during analysis. The method is similar to external calibration in that a series of standards is needed and similar to standard additions in that a known amount of a standard is added. The method involves addition of a known quantity of a reference compound, the internal standard, in all the standards and the unknown samples analyzed for quantification. The response signals for both the analyte and the internal standard are measured [103].

The concentration ratio and the response ratio of analyte to internal standard are calculated for each solution. A calibration plot of the response ratio versus concentration ratio is prepared. The response ratio is not affected by a variation in the response signals that may vary from sample to sample, resulting in a linear plot over a certain range of ratios. Linear regression is performed on the resulting plot and a linear least squares (LLS) equation is obtained. After the response ratio for the unknown is determined, the LLS equation of the internal standard line is used to calculate the corresponding concentration ratio. The known concentration of the internal standard added is multiplied by the ratio to obtain the concentration of the unknown [103].

The internal standard method is ideal for analysis without auto-sampler equipment as it is independent of the accuracy of injection. A suitable compound is necessary to ensure the success of the internal standard method. The requirements that need to be met by such a compound are that it is not present in the unknown, it does not react with anything in the sample matrix, both the analyte and the internal standard are affected in the same manner and show similar changes in the instrument response. Lastly, the analyte and internal standard should produce separate signals, which do not overlap each other or the signals of matrix components. The internal standard method is primarily associated with instrumental methods having sharp, narrow output signals, such as mass spectrometry [103].

Considering all the characteristics, the internal standard method is the most promising method in the quantification of the urine metabolites by DI-ESI-MS, provided a suitable internal standard compound can be identified, and was applied in the quantification of the urine metabolites: creatinine, creatine and caffeine in this work. 1-Methyl-3-phenylpropylamine was used as a primary internal standard to quantify creatinine. After the concentration of creatinine has been determined,

creatinine acts as the secondary internal standard to quantify creatine and caffeine. The quantification was achieved by generating calibration curves for the metabolites.

2.4 Proposed method development strategy

A useful strategy towards developing a practical method for the simultaneous detection and quantification of urine metabolites involved a close study of their chemistry, particularly their capacity for ionization and ability to form hydrogen bonds in solution. The chemical behavior of these compounds was evident from their mass spectra.

Based on some fundamentals of different methods of quantification and the preliminary observations, discussed in chapter 3, key parameters were established which allow direct infusion mass spectrometry to be used as proposed. Firstly, to ensure low viscosity and volatility of the carrier solvent, methanol was incorporated in the solvent mixture. Since the metabolites to be analyzed are highly polar, water was also incorporated. To promote quantitative ionization of the analyte molecules into $[M+H]^+$ species, acetic acid was also made part of the solvent carrier for the proposed DI-ESI-MS method. The fragmentation voltage which is applied at the skimmer, which is part of the ion transfer optics, was used to ensure that the analyte molecules are in the monomeric form and not clusters. This is important as it prevents multiplicity of the signals. Furthermore, an internal standard method was selected for DI-ESI-MS in view of the associated high level of signal intensity variations. The internal standard method was most appropriate in another sense as well. The internal standard method allows use of an endogenous metabolite, creatinine, as an internal standard for quantitative determination of other urine metabolites. To use creatinine successfully as an internal standard, a primary standard is required, which is not a component of urine, so that creatinine concentration can be determined against it. For this purpose, 1-methyl-3-phenylpropylamine was selected.

Chapter 3: Results and discussion

3.1 Preliminary observations

Preliminary LC–MS analysis of creatinine (11 µg/mL in deionized water) revealed that creatinine was converted online to creatine, and indicated the presence of sodium adducts of both compounds (Figure 3.1). A Waters LC–MS Q–TOF instrument used in these preliminary experiments was equipped with an atmospheric pressure chemical ionization (APCI) chamber, as shown in Figure 3.2, which could facilitate online conversion of creatinine to creatine. More importantly the Waters LC–MS instrument uses the conventional direct electrospray process for ionization where a high positive voltage (2500 to 4500 V) is applied to a metal capillary. In this set-up it has been reported that electrochemical reactions may occur which could lead to bond cleavage and hydrolysis. Such a process would convert creatinine into creatine. Conventional, direct electrospray ionization mass spectrometry was therefore not pursued any further in this work.

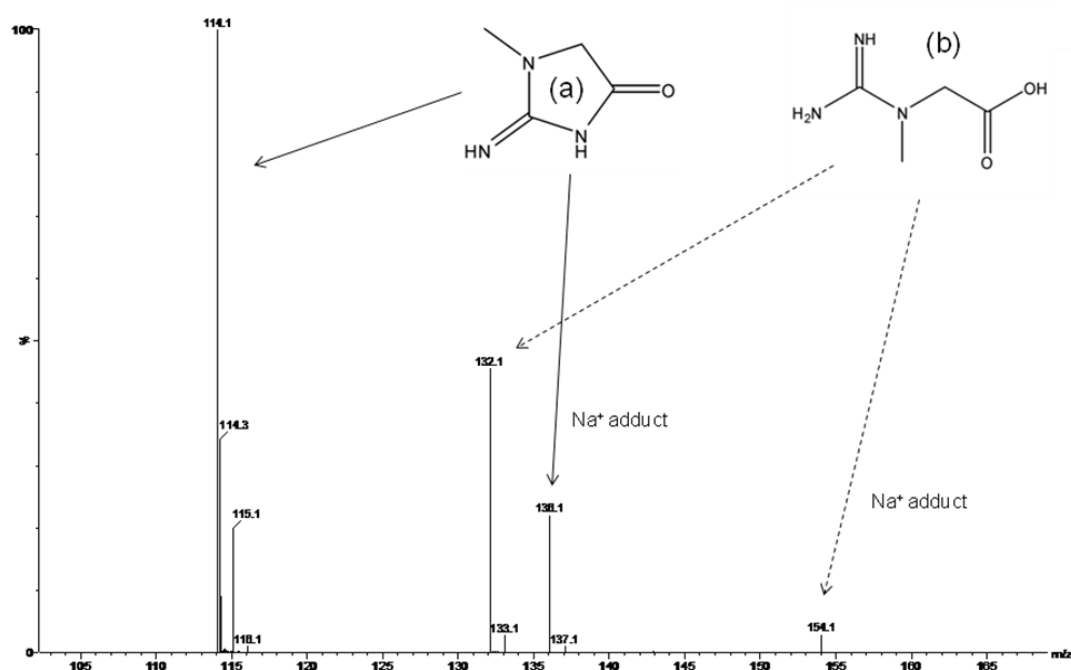


Figure 3.1. Mass spectrum of creatinine with APCI as the method of ionization. Online conversion of creatinine (a) to creatine (b) and sodium adduct formation with both compounds are observed.

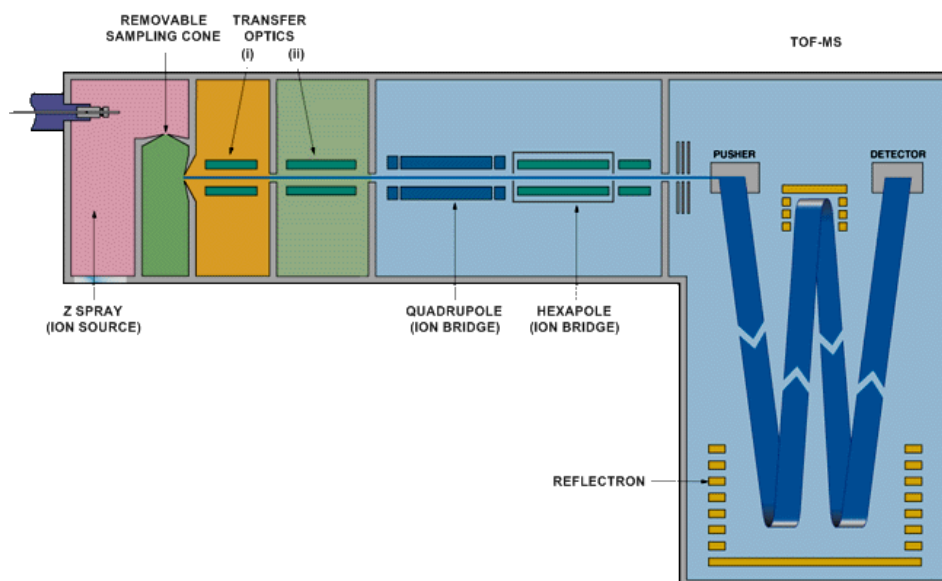


Figure 3.2. Waters LC-MS Q-TOF Mass Spectrometer.

Further experiments were carried out on the Agilent 6120 LC-MS system which is equipped with a pneumatically assisted atmospheric pressure ESI chamber as shown in Figure 2.1. When creatinine (10 $\mu\text{g/mL}$ in 90:10 (% v/v) methanol and water) was analyzed, online conversion of creatinine to creatine was not observed. The mass spectrum is shown in Figure 3.3. Considering the multiple peaks in the mass spectrum which were generated from a single analyte, it was evident that the response of creatinine needed to be simplified. As shown in Figure 3.3, four creatinine species $[\text{M}+\text{H}]^+$, $[\text{M}+\text{Na}]^+$, $[\text{2M}+\text{H}]^+$ and $[\text{2M}+\text{Na}]^+$ with m/z of 114, 136, 227 and 249 respectively, were observed instead of a single peak, $[\text{M}+\text{H}]^+$ at 114 m/z .

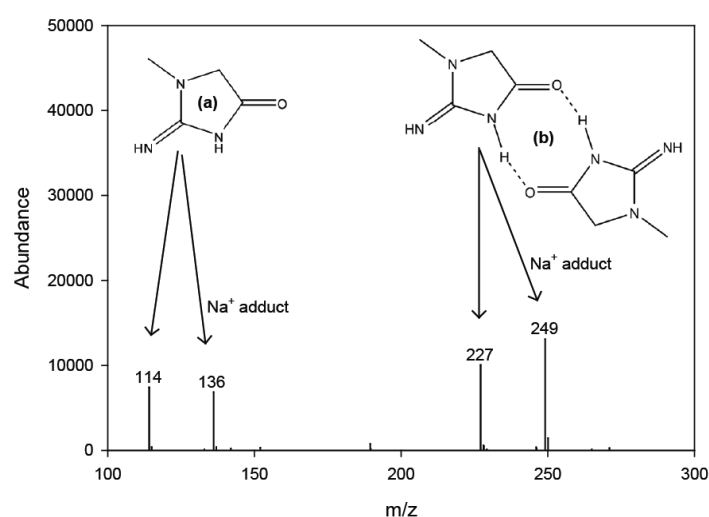


Figure 3.3. Mass spectrum of creatinine with atmospheric pressure ESI as ionization method.

Online conversion of creatinine (a) to creatine is not observed. Creatinine sodium adduct formation occurred and dimerization (b) of both creatinine and the sodium adducts are observed.

3.2 Overcoming sodium adduct ion formation

Although all measures were taken to ensure contamination free equipment were used, sodium adduct ions were observed. It is possible that adventitious sodium ions, likely from storage bottles and vials or residues due to handling, compete with hydrogen ions in pH-neutral solutions and form undesirable sodium adducts [104-106]. The solvent in the creatinine solution consisted of 90:10 (% v/v) methanol and water. Acetic acid was added in order to promote the formation of hydrogen-adduct $[M+H]^+$ ions over sodium adduct $[M+Na]^+$ ions. When a new creatinine solution (10 $\mu\text{g/mL}$) was made up with methanol, water and acetic acid in 90:9.9:0.1 (% v/v/v) ratio and analyzed, signal intensities of the two creatinine sodium adduct species, $[M+Na]^+$ and $[2M+Na]^+$, were significantly reduced (Figure 3.4).

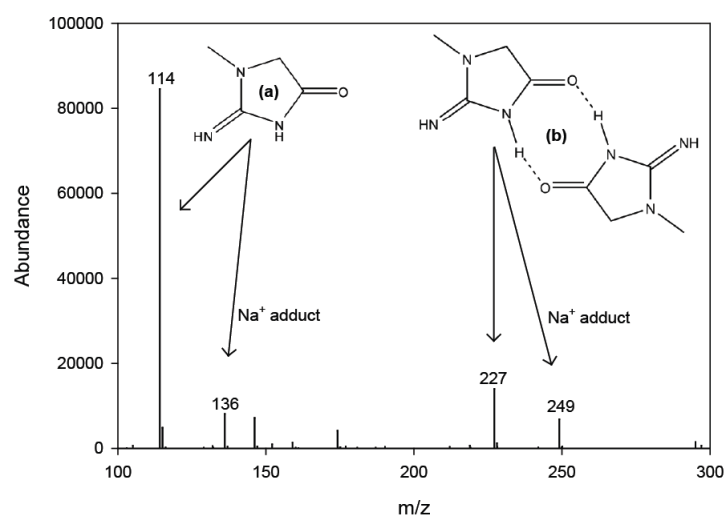


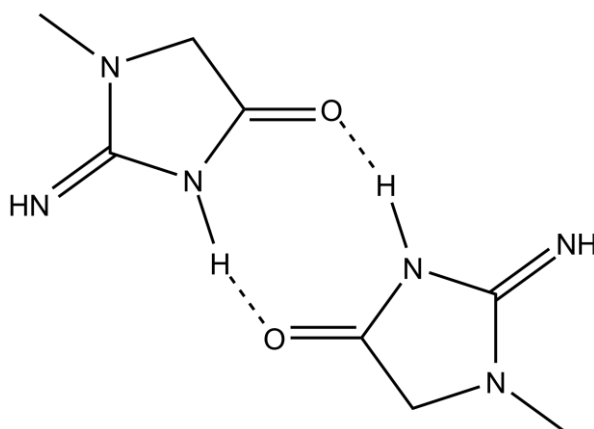
Figure 3.4. Mass spectrum of creatinine with the addition of acetic acid to the solvent. (a) Creatinine; (b) creatinine dimer. The availability of an excess amount of hydrogen ions shadow the sodium ions present and a reduction in the $[M+Na]^+$, $[2M+H]^+$ and $[2M+Na]^+$ species is observed.

Although a significant decrease in the creatinine dimer species, $[2M+H]^+$ and $[2M+Na]^+$, was observed after addition of acetic acid, further reduction of dimer formation required additional measures as discussed next.

3.3 Overcoming creatinine dimerization

Creatinine molecules, as well as creatine, seem to form hydrogen bonded dimers in solution (Scheme 3.1), which survive transition into the gas phase as $[2M+H]^+$ ions. The intermolecular H-bonds are generally weaker than intramolecular bonds and are disrupted by high voltage applied at

the skimmer. Voltage applied at this point of the ion transmission optics is referred to as fragmentation voltage as it can cause fragmentation of ordinary compounds when it is set sufficiently high.



Scheme 3.1. Proposed chemical structure for a creatinine dimer. Two hydrogen bonds are formed between two creatinine molecules to form the dimer.

An experiment was conducted whereby fragmentation voltage was varied in small 5 V increments to identify the optimum setting at which the maximum amount of creatinine as well as creatine ions remain intact, while the maximum amount of dimer ions $[2M+H]^+$ and $[2M+Na]^+$, is fragmented into monomer ions $[M+H]^+$. Creatinine solution (10.0 $\mu\text{g/mL}$ in 90:9.9:0.1 (% v/v/v) methanol, water and acetic acid) was analyzed at different fragmentation voltages which were increased in 5 V increments from 70 V to a final value of 120 V. A significant decrease in the dimer species, $[2M+H]^+$ and $[2M+Na]^+$, was observed as shown in Figure 3.5.

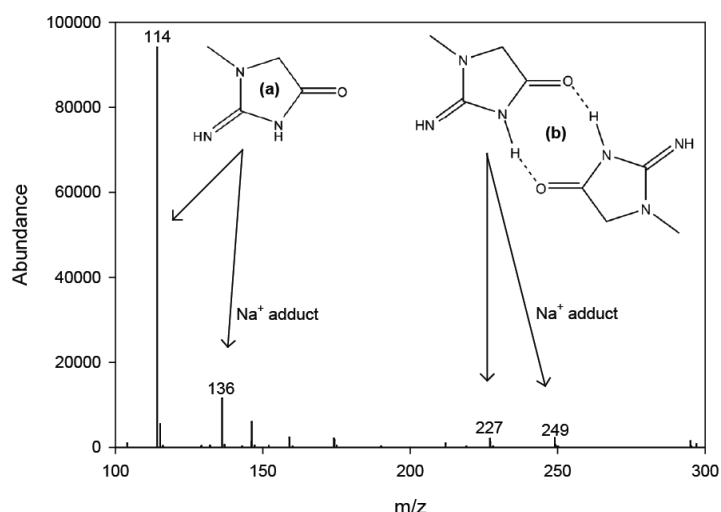


Figure 3.5. Mass spectrum of creatinine. (a) Creatinine; (b) creatinine dimer. The reduction in the $[2M+H]^+$ and $[2M+Na]^+$ species observed when fragmentation voltage was set at 95 V.

A comprehensive picture of what happens to each of the four creatinine ions $[M+H]^+$, $[M+Na]^+$, $[2M+H]^+$ and $[2M+Na]^+$ and those of creatine $[M+H]^+$ is reflected in the plot of fragmentation voltage against signal intensity (Figure 3.6).

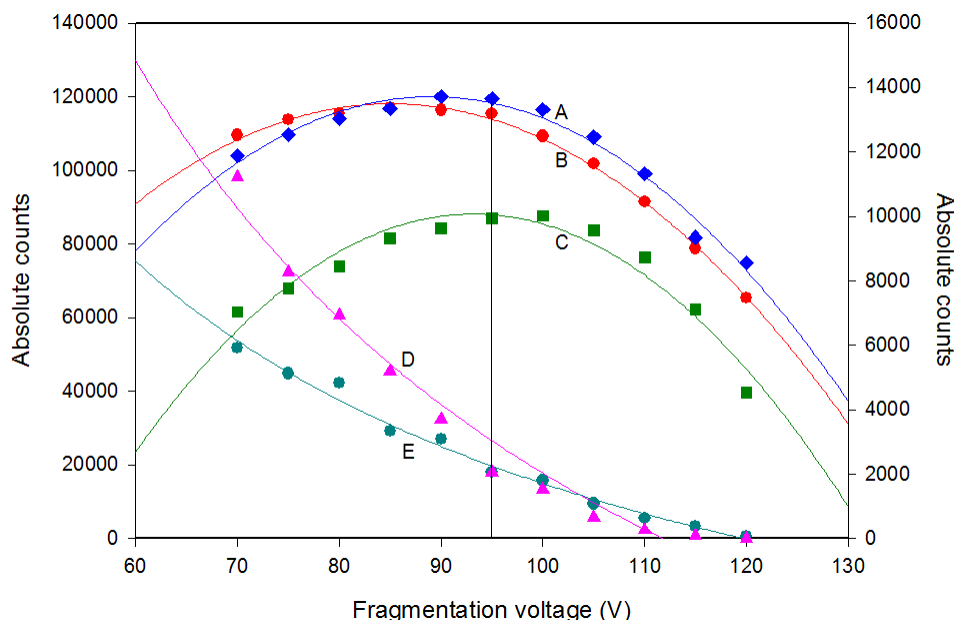


Figure 3.6. Change in signal intensity of urinary metabolites with increase in fragmentation voltage. (A) Creatine, 132 m/z (—◆—); (B) Creatinine, 114 m/z (—●—); (C) Creatinine sodium adduct, 136 m/z (—■—); (D) Creatinine dimer, 227 m/z (—▲—); and (E) Creatinine dimer sodium adduct, 249 m/z (—●—). A and B are plotted on the left y-axis and a quadratic regression of both resulted in R^2 values of 0.997 and 0.977 respectively. C, D and E are plotted on the right y-axis. A quadratic regression of C resulted in a R^2 value of 0.932. Inverse first order regression of D and E resulted in R^2 values of 0.970 and 0.987 respectively. All data points represent averages of duplicate values.

The hydrogen bonds between the creatinine monomers that form the creatinine dimer species, $[2M+H]^+$ and $[2M+Na]^+$, are broken by the fragmentation voltage. An increase in creatinine and creatine ions was clearly seen when fragmentation voltage was increased from 70 V to about 90 V. However, further increase to greater than 95 V appeared to destroy the monomers. The optimum fragmentation voltage was identified as 95 V where a substantial decrease in the dimer species was obtained without significant loss in monomer species.

3.4 The carrier solvent

The development of a direct infusion mass spectrometry method suited to quantify the urinary metabolites required the selection of appropriate solvents. The solvents influence the ability of the

analyte to enter the nebulizer capillary and the ESI mechanism. The latter has an effect on the ion transportation, ion separation and ion detection [104] which was discussed in detail in subsection 1.5.1.

The inlet where the sample is introduced into the capillary of the nebulizer posed certain challenges. The sample introduction depended solely on the gravitational force on the analyte as a pump was not used. The viscosity of the sample determines if the sample will be able to enter the capillary of the nebulizer. A solvent containing methanol, water and acetic acid was used in various ratios. All the solvent solutions contained 90% methanol and either 9.9% and 0.1% water and acetic acid, respectively, or 7.5% and 2.5% water and acetic acid, respectively. The methanol ensured a low viscosity and the addition of acetic acid was discussed in section 3.2.

As mentioned, ESI is influenced by the composition of the solvent. Positive ion ESI is usually performed with protonated solutions such as methanol/water. The solvent reacts with a weak acid, in this case acetic acid, which results in the charge being carried by protonated solvent clusters. Protonation of the analyte is facilitated by the low pH of the acidic solution [104].

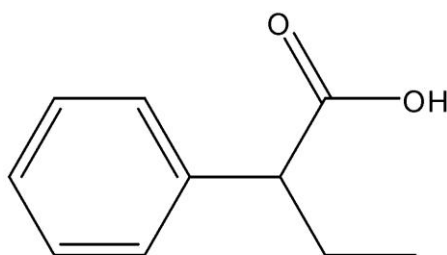
3.5 The primary standard

The need for a reference compound as a primary internal standard was discussed in section **Error! Reference source not found.** Two compounds were identified and investigated as possible candidates for this purpose. It has been reported in the literature that both 2-phenylbutyric acid (PBA) [107] and 1-methyl-3-phenylpropyl-amine (MPPA) [108-111] are suitable internal standards when analyzing urine or other humic samples with analytical methods such as HPLC and GC-MS, although both have not specifically been reported as reliable internal standards when analyzing urine samples with direct infusion mass spectrometry.

3.5.1 2-Phenylbutyric acid

The PBA was easier to use because it is available as a powder (Scheme 3.2). It has a molecular weight of 164.2 g/mol. The PBA met most of the criteria for an internal standard, namely it is not present in urine, it does not react with anything in the sample matrix, and both the analyte and PBA are affected in the same manner and leads to similar changes in the instrument response. Furthermore, the analyte and internal standard produce separate signals, which do not overlap each other or the

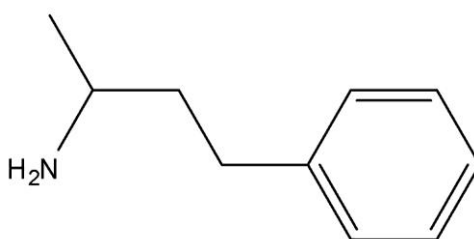
signals due to matrix components. However, mass spectrometry analysis showed that PBA is not readily protonated and prefer to undergo deprotonation of its acidic moiety. PBA could be analyzed in the negative mode with a mass spectrometer, but a more suitable internal reference that could be readily protonated and analyzed in the positive mode was preferred. The second compound, MPPA, was therefore investigated.



Scheme 3.2. Chemical structure for PBA. PBA is not easily protonated.

3.5.2 1-Methyl-3-phenylpropylamine

The MPPA is only available as a liquid (Scheme 3.3). The structure of MPPA was considered and the protonation of the amine group is possible. It met all the requirements for an internal standard as described in the previous subsection.



Scheme 3.3. Chemical structure for MPPA. The amine group allows easy protonation which makes MPPA better suited as a primary internal standard in positive mode MS analysis.

MPPA has a molecular weight of 149.23 g/mol and is thus comparable to all the urine metabolites of interest, namely creatinine, creatine and caffeine. Mass spectrometry analysis of MPPA confirmed that it is protonated and thus suitable to act as a primary internal standard.

3.6 Calibration curves

Initially, a study was conducted to establish if creatinine can be employed as a secondary internal standard. Creatine was the analyte in unknown quantities. Standards of creatine were prepared and 5 $\mu\text{g/mL}$ of the internal standard was added to each of the standards. The standards covered a range of concentrations similar to that expected to be found in urine to construct a calibration curve for creatine. Determination of the resulting response signals allowed for a calibration curve of creatine to be obtained over a final concentration range of 1–10 $\mu\text{g/mL}$ (Figure 3.7). The result indicated a linear calibration curve for creatine which was expected since the internal standard method corrects for any instrumental response factors.

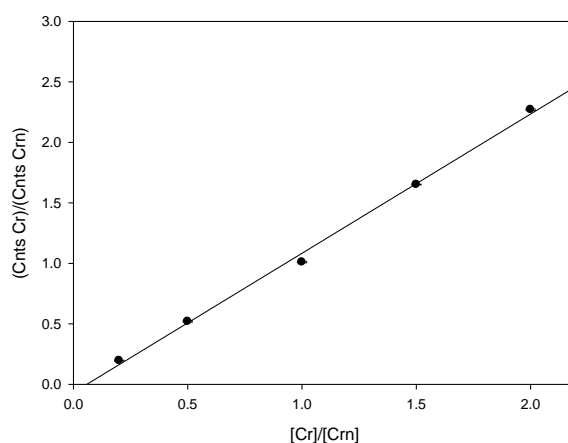


Figure 3.7. Calibration curve of creatine. Linear regression of the plot resulted in a R^2 value of 0.997. All data points represent the mean of duplicate values, with the error bars denoting the standard deviation.

The results from this experiment suggested that the quantification of urine metabolites by direct infusion mass spectrometry is possible. Standards of creatinine and caffeine were prepared and 5 $\mu\text{g/mL}$ of the primary internal standard, MPPA, and the secondary internal standard, creatinine, were added to the respective sets of standards. The standards covered a range of concentrations similar to that expected to be found in urine, taking into account the dilution that occurs during sample preparation, to construct a calibration curve for creatinine and caffeine. Determination of the resulting response signals allowed for calibration curves of both metabolites to be obtained over a final concentration range of 1–10 $\mu\text{g/mL}$ (Figure 3.8). The results indicated linear calibration curves for creatinine and caffeine.

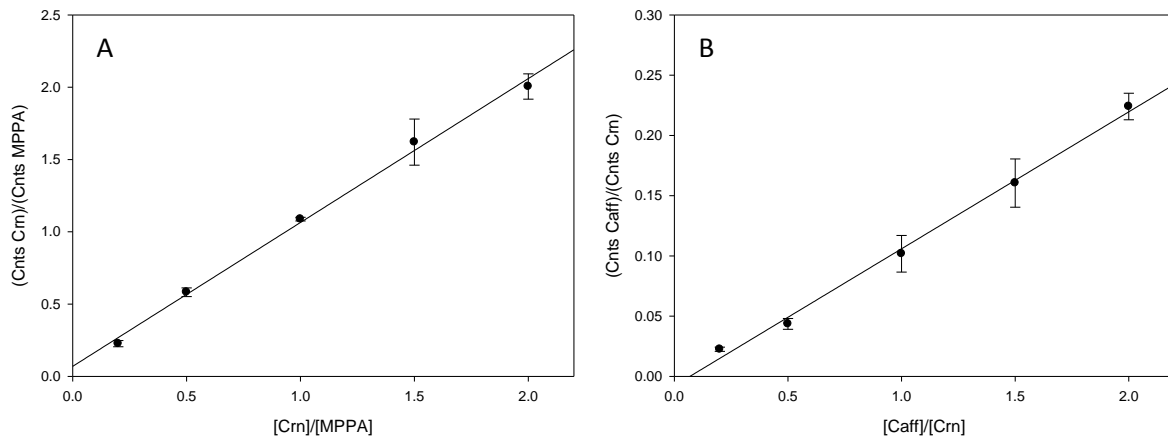


Figure 3.8. Calibration curves of the urinary metabolites. (A) Creatinine calibration curve; (B) Caffeine calibration curve. Linear regression of A and B resulted in R^2 values of 0.985 and 0.977 respectively. All data points represent the mean of triplicate values, with the error bars denoting the standard deviation.

3.7 Limits of detection and quantification

The ability of the developed method to accurately detect and quantify the urine metabolites in artificial samples was subsequently assessed in terms of the limit of detection (LOD) and limit of quantification (LOQ). The LOD of a specific analyte is defined as the lowest concentration of the analyte that can be detected that is statistically different from an analytical blank. The definition of LOQ is the lowest concentration of a specific analyte which can be quantitatively analyzed with reasonable reliability. LOD and LOQ are calculated by estimating the LOD from previous experience with the method. A sample containing approximately the same concentration ($1 \mu\text{g/mL}$) as the LOD is prepared and the signal measured for six replicate samples ($n = 6$). The standard deviation (s_d) of the six measurements is computed by using the following equation,

$$s_d = \sqrt{\frac{\sum_i (x_i - \bar{x})^2}{n-1}} \quad \text{Eq. 3}$$

where n is the number of measurements, x_i is the value of each measurement and \bar{x} is the mean of n measurements.

The signal from six blanks (containing no analyte) is measured and the mean value, y_{blank} , calculated. By using equation 4 to determine the signal detection limit (y_{dl}), this procedure produces a y_{dl} with a 99% chance of being greater than the blank. Once the y_{dl} and signal quantification limit (y_{ql}) are known, the LOD and LOQ can be calculated and expressed in concentration units.

$$y_{dl} = y_{blank} + 3s_d \quad \text{Eq. 4}$$

$$LOD(\mu\text{g} / \text{mL}) = \frac{y_{dl}}{\bar{x}} \quad \text{Eq. 5}$$

$$y_{ql} = y_{blank} + 10s_d \quad \text{Eq. 6}$$

$$LOQ(\mu\text{g} / \text{mL}) = \frac{y_{ql}}{\bar{x}} \quad \text{Eq. 7}$$

The LOD and LOQ values for all the urinary metabolites of interest were calculated in this manner and the results are shown in Table 3.1.

Table 3.1. LOD and LOQ values for the urinary metabolites of interest.

Compound	s_d	y_{blank}	y_{dl}	y_{ql}	\bar{x}	LOD $\mu\text{g}/\text{mL}$	LOQ $\mu\text{g}/\text{mL}$	Analysis range $\mu\text{g}/\text{mL}$
Creatine	326.6	1369	2349	4635	32239	0.073	0.144	1 – 10
Creatinine	1160	438.7	3917	12034	6559	0.597	1.835	1 – 10
Caffeine	141.9	61.2	486.8	1480	341.0	1.427	4.339	1 – 10

LOD and LOQ are very strict measurements of accuracy and an analytical method that gives very low variability in the obtained values is expected to produce satisfactory values.

3.8 Quantification of artificial samples

The ability of the developed method to accurately detect and quantify the creatinine and caffeine as the urinary metabolites was evaluated. The primary internal standard, MPPA, was added to three artificial samples containing an unknown amount of creatinine and analyzed. The concentration of creatinine and the percentage recovery were successfully calculated for each sample using the LLS equation of the creatinine calibration curve in Figure 3.8. Creatinine as secondary internal standard

was used to successfully calculate the concentration and the percentage recovery of caffeine for each sample using the LLS equation of the caffeine calibration curve in Figure 3.8. The results are reported in Table 3.2.

Table 3.2. Quantitative results for creatinine and caffeine samples with unknown amounts. All the data represent the mean of triplicate values, with the standard deviation noted.

Compound	Sample	Theoretical concentration $\mu\text{g/mL}$	Measured concentration $\mu\text{g/mL}$	% recovery
Creatinine	Unknown 1	67.5	68.4 \pm 3.1	101 \pm 3.6
	Unknown 2	22.5	22.4 \pm 0.9	99.6 \pm 7.1
	Unknown 3	15.0	15.3 \pm 2.3	102 \pm 3.8
Caffeine	Unknown 1	85.0	82.5 \pm 1.4	97.1 \pm 2.1
	Unknown 2	12.5	12.3 \pm 1.1	98.4 \pm 4.7
	Unknown 3	60.0	60.5 \pm 0.8	101 \pm 5.1

3.9 Quantification of urine sample

A urine sample was analyzed using the DI-ESI-MS method to detect and quantify creatinine and caffeine. The internal standard, MPPA, was added and a final dilution of 100-fold obtained. The resulting mass spectrum of the analyzed urine sample is shown in Figure 3.9.

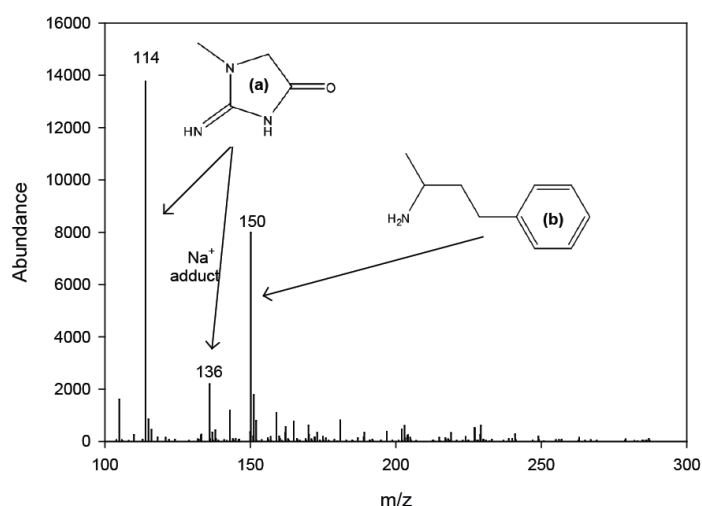


Figure 3.9. Mass spectrum of a urine sample diluted 100-fold. Creatinine (a), MPPA (b) and the sodium-adduct of (a) are observed at m/z of 114, 150 and 136, respectively. Caffeine is not observed at 195 m/z .

The mass spectra confirmed the presence of creatinine and MPPA at m/z of 114 and 150, respectively. The creatinine sodium adduct was also present at 136 m/z . Creatinine was quantified and a concentration of $835 \pm 3.1 \mu\text{g/mL}$ was obtained. The signal for caffeine (195 m/z) was very low and many ions due to the urine matrix are observed. The need to dilute urine 100-fold to analyze creatinine in the calibration range proved to be detrimental to the determination of caffeine due to the low concentration present in the sample. Ionizability of caffeine is a problem even in other systems that use it as a calibrant. Analysis of an undiluted urine sample to confirm the presence of caffeine was unsuccessful due to the high salt and protein concentrations present which clogged the spray capillary rendering ESI impossible. Therefore we were unable to perform simultaneous quantification for both compounds in a urine sample.

Chapter 4:

Method validation – Comparison with HPLC-based quantitation of creatinine and caffeine

4.1 Introduction

High performance liquid chromatography (HPLC) is a popular and relatively simple and sensitive analytical method commonly used to analyze various substances. It is a fast, reproducible and robust form of liquid chromatography which often is highly automated. HPLC in conjunction with a range of detectors has become one of the main tools of a modern laboratory.

However, in contrast to gas chromatography (GC), which was successfully hyphenated with mass spectrometry (MS) in the early 1960s and has for long been the golden standard in analytical toxicology, the hyphenation of liquid chromatography (LC) with MS took more than 30 years longer [112, 113]. At first the scientific and/or practical progress of hyphenating LC with MS was strained by using existing LC–UV or GC–MS procedures which had many limitations for this new technique, including poor spectral information (in single-stage apparatus), poor ionization reproducibility, and susceptibility to matrix effects (ion suppression or enhancements). These limitations of LC–MS have in recent years experienced considerable improvements including instrumental advances, the development of suitable specimen clean-up procedures to avoid matrix effects, improvements of reagents and in chromatographic separation, as well as effective internal standard quantification [61].

Electrospray ionization (ESI) was discussed in subsection 1.5.1 and is the ionization method most widely employed in LC–MS. In ESI, direct analysis of analyte solutions is made possible by ionization at atmospheric pressure. HPLC and MS do, however, present some problems that arise from their intrinsically different operative conditions. LC employs (often non-volatile) mobile phases to achieve high chromatographic resolution and mass spectrometers generally exhibit a low tolerance for the high liquid flows used in LC. Further complications in LC–MS operation stems from the differences among classes of analytes with regard to molecular weight, polarity and stability; the variable solvent composition of the mobile phase reflecting on the formation of droplets of different

dimension and lifetimes; and the compatibility of the chromatographic eluate flow with the sprayer operative flow [114].

Despite these constraints, LC–MS is nowadays becoming a routine technique ideally suited most notably for quantification of polar, thermolabile, or low-dosed drugs due to its ability to guarantee the stability of analytes in the biosample during the whole analytical procedure [60, 61, 114, 115]. However, it is important to bear in mind several aspects when establishing LC–MS screening procedures in routine work. The spectral information attainable by electron impact mass spectra in GC–MS can be reached in LC–MS only by using product ion spectrum mode on tandem MS instruments, whereas the information of single-stage ESI and/or atmospheric pressure chemical ionization (APCI) spectra is rather poor. In-source fragmentation caused by increasing the fragmentor voltage in ESI and/or APCI allows the formation of structure-related fragments. Traces with different fragmentor voltages can be recorded during LC separation using modern apparatus which allow very fast switching between voltages. The loss of spectral information can thus be limited when analyzing compounds with different fragmentation properties in one run [116–118]. A second important consideration is the fact that LC–MS is susceptible to matrix effects, with the ionization source determining the extent thereof. APCI is less susceptible to matrix effects than ESI, although the sensitivity is generally lower, depending on the analyte, matrix and the instrument used and should always be evaluated [119]. In comparison, LC–MS is much more selective and sensitive than LC–UV, although the latter should be free of matrix interferences.

The importance of LC–MS in routine quantitative clinical and toxicological analysis of various analytes has increased in the past 15 years and has certainly left the development stage, especially considering single-stage or tandem LC–MS/MS with ESI or APCI [60, 120]. These LC–MS techniques have made it possible to explore new ways to interpret clinical and toxicological data. A few examples include the determination of chronic alcohol consumption by determining ethanol conjugates in urine, plasma and hair [121], and sensitive detection of chemical agents in hair in the case of drug-facilitated crimes [122].

Several HPLC methods have been described for the analysis of urinary creatinine either alone or in conjunction with various other target analytes. These HPLC methods include the use of reversed-phase LC on a range of different stationary phases, ion-pair chromatography, automatic analyzers using cation-exchange columns and more recently hydrophilic interaction chromatography (HILIC) as reviewed in section 1.4 [20, 30, 48, 123].

Reversed-phase HPLC is a powerful separation technique; however the low retention and poor separation of polar molecules are major limitations [123, 124]. Most hydrophilic compounds elute at or near the column void volume under typical reversed-phase conditions using conventional C18 columns and highly aqueous mobile phases [125]. Traditionally, some polar compounds have been separated with non-aqueous mobile phases using normal-phase liquid chromatography (NP-LC). A difficulty does arise in dissolving hydrophilic compounds in these phases, resulting in an important limitation [123].

HILIC presents a viable alternative to reversed-phase, ion-exchange and normal-phase LC for the separation of such polar compounds with the most important reasons being the clear advantages with regard to the solubility of biological compounds and no need for compound derivatization. These advantages facilitate easier sample processing and compatibility with further mass spectrometric analysis, where volatile buffers are used and a low amount of volatile salt is allowed [123].

HILIC is similar to normal-phase chromatography because polar compounds are retained longer than the non-polar ones and the polar component of the mobile phase (usually water) is the strong solvent. Retention of the polar compounds is achieved by the use of a polar stationary phase in HILIC and an initial mobile phase consisting of a high content of an organic solvent. Water in the mobile phase forms a stagnant enriched aqueous layer on the polar stationary phase surface. Analytes may selectively partition into this layer as described by Alpert [126], and more recently evaluated and confirmed by McCalley and Neue [127]. This allows for the promotion of hydrophilic interactions between the analyte and the polar hydrophilic stationary phase. The nature of the mobile phases used in HILIC is comparable to reversed-phase separations, which results in HILIC being superior to normal-phase chromatography with regard to the solubility of analytes in the mobile phase and matrix compatibility [123, 128].

4.2 Strategy

The aim of this part of the study was to develop a LC-UV-MS method for the validation of the developed DI-ESI-MS method reported in the two previous chapters. Separation with LC should reduce matrix effects that could affect the results obtained with the DI-ESI-MS method. A comparison between the DI-ESI-MS method and the LC method with either UV or MS detection is

therefore needed to determine if significant differences are obtained in terms of a) precision, and b) the mean values produced by these methods for validation purposes. Separation with LC should reduce matrix effects that can affect the results obtained with the DI-ESI-MS method. The structures of creatinine and caffeine were considered in the development of a LC-UV-MS method for the separation, detection and quantification of these metabolites. The aspects that were considered are discussed in detail below.

4.2.1 Development of LC-UV-MS method

Inspection of the chemical structures and properties of the two metabolites, creatinine and caffeine, and the internal standard, MPPA, highlights the parameters that must be considered in the development of a suitable HPLC separation method.

The variable number of polar covalent bonds among the two metabolites and the internal standard accounts for the most important determinant of differential polarity and hydrophobicity between them. These differences in polarity and hydrophobicity can be quantified by calculation of the specific partition constant (P) for each compound [129]. Although commonly used for this purpose, the log of the calculated octanol/water partition constant, $c \log P$, is only valid for neutral compounds. Evaluation of the pK_a values of the target molecules reveals that, at a pH of 4.5, all three the compounds are to some extent charged due to their respective basic or acidic functional groups. The distribution of these compounds in octanol/water mixture therefore becomes pH-dependent. The distribution coefficient, D , serves as a quantitative descriptor of hydrophobicity for ionizable compounds. Calculation of D depends on the assumption that only the neutral form of a compound will partition into the organic phase [130, 131]. The log of D can be calculated from $c \log P$ and pK_a values according to equation 8,

$$\log D = c \log P - \log(1 + 10^{(pH - pK_a)\Delta}) \quad \text{Eq. 8}$$

where Δ is equal to 1 for acids and -1 for bases.

Ionization of the compounds decreases their retention in reversed-phase chromatography, whereas it increases their retention when using HILIC. The pK_a , $c \log P$ and $\log D$ values are presented in Table 4.1 for the two metabolites and the internal standard under consideration. Hydrophobicity is directly

proportional to $c \log P$ and $\log D$ values, i.e. the larger the $c \log P$ and $\log D$ value, the more hydrophobic the compound.

Table 4.1. Physical properties of two metabolites and internal standard.

Physical property	Compound		
	Creatinine	Caffeine	MPPA
pK_a^a	4.8	10.4	9.79
$c \log P^b$	-1.76	-0.07	2.12
$\log D^c$	-2.75; -2.45	-0.63	-0.84

^a pK_a values were obtained from the PHYSPROP@Database, S.R.C.

^b $\log P$ values were obtained from ChemDraw and calculated using the embedded CLOGP function developed by the BioByte Corporation.

^c $\log D$ values were obtained from SciFinder and calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V11.02 (© 1994-2012 ACD/Labs) at pH 4 (first value) and pH 5 (second value). If the $\log D$ values are the same value at both pH values, it is reported only once.

The $c \log P$ and $\log D$ values of the two metabolites and the internal standard exhibit good variation, indicating that their separation by reversed-phase LC and/or HILIC, in which the compounds would elute either in order of increasing or decreasing hydrophobicity respectively, should be possible. This suggests both reversed-phase LC and HILIC as effective methods for the separation of the two metabolites and the internal standard.

Two types of detectors, UV and MS, were selected for the development of the HPLC method. UV is an established method for the routine quantification of compounds, provided that sufficient selectivity and sensitivity is attainable. MS detection on the other hand is most commonly used as a qualitative method for identification of compounds of interest. The same optimized experimental conditions as reported for the DI-ESI-MS method were used for LC-MS detection. LC-MS also allows the generation of extracted ion chromatograms, which were evaluated for quantitative analysis in this work.

4.2.2 Metabolite quantification

The quantification of the two metabolites from chromatographic peak areas can be achieved by following different approaches. Three common methods of quantification, namely the external standard, standard addition and the internal standard method can be used in HPLC for validation of the direct infusion mass spectrometry method. The internal standard method was selected for

validation by HPLC because this approach was used in the development of the direct infusion mass spectrometry method and because the HPLC instrument used was equipped with a manual injector.

4.3 Materials and experimental procedures

4.3.1 Chemicals and reagents

All chemical and chromatographic reagents used were of HPLC grade. Creatinine, caffeine, MPPA, methanol (MeOH), ammonium acetate, ammonium formate, acetic acid and formic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Acetonitrile (ACN) was purchased from ROMIL Pure Chemistry (Cambridge, UK). Millipore water (Millipore, Milford, Massachusetts, USA) was used in the preparation of all solutions.

4.3.2 Materials and instrumentation

Standards were used to develop the method. Separations were performed in a HPLC Agilent 1220 Infinity LC system (Agilent Technologies, Inc., Santa Clara, California, USA) equipped with a gradient pump (Model # G4281B) with an integrated degassing unit, and a manual injector. Ultraviolet detection was performed using a Model # G4284B variable wavelength detector, which was used in the range of 205–260 nm. Chromatograms were recorded and processed using Agilent ChemStation software for LC, Rev. B.04.03. The ZORBAX® SB-C18 (2.1 × 50 mm, 1.8 µm) reversed-phase column was obtained from Agilent Technologies and the BETASIL® Diol-100 (4.6 × 250 mm, 5 µm) HILIC column was obtained from Thermo Electron Corporation (Waltham, Massachusetts, USA). The BETASIL® Diol-100 column was protected by an aminopropyl SecurityGuard™ (3 × 4 mm, 5 µm) column obtained from Phenomenex, Inc. (Torrance, California, USA).

pK_a values were obtained from the PHYSPROP® Database, S.R.C. (North Syracuse, New York, USA). Calculation of $c \log P$ values for all compounds was performed in ChemDraw® (CambridgeSoft, Cambridge, Massachusetts, USA) using the embedded CLOGP function. CLOGP software calculates $c \log P$ values based on fragmental contributions and was developed by the BioByte Corporation (Claremont, California, USA). $\log D$ values were obtained from SciFinder and calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V11.02 (© 1994-2012 ACD/Labs). All graphical and statistical analyses were performed with SigmaPlot® 11.0 (Systat Software, Inc., Chicago, Illinois, USA).

4.3.3 Procedures for LC–UV–MS method development

4.3.3.1 *Experimental conditions for the reversed-phase column*

The ZORBAX® SB-C18 column was evaluated for separation of creatinine, caffeine and MPPA standards. A binary solvent system was used that consisted of 5 mM ammonium acetate (pH 4.5) in water and ACN in a 99:1 (% v/v) ratio. A 30 min isocratic analysis with a flow rate of 0.250 mL/min was used to analyze a 2 µL sample injection containing 10 µg/mL creatinine, caffeine and MPPA dissolved in 90:7.5:2.5 (% v/v/v) methanol, water and acetic acid. Detection was performed at 250 nm. The analysis was repeated at a flow rate of 1.000 mL/min in a second experiment, which was also repeated with detection performed at 230 nm.

4.3.3.2 *Experimental conditions for the HILIC column*

The BETASIL® Diol-100 column was evaluated and used for the development of an extended method for the separation of creatinine, caffeine and MPPA standards. A binary solvent system was used that consisted of 5 mM ammonium acetate (pH 4.5) in water and ACN, respectively. Initially a 12 min analysis method (Method A) with a flow rate of 1.000 mL/min was used to analyze a 2 µL sample injection containing 10 µg/mL creatinine, caffeine and MPPA dissolved in pure water. The method started with 99% ACN and 1% ammonium acetate, after which the percentage of ammonium acetate was increased over time as follows: isocratic, 0-2 min; 1-50% ammonium acetate, 2-10 min; isocratic, 10-11 min; 50-1% ammonium acetate, 11-12 min. A 5 min post-run ensured the system was at initial conditions. Detection was performed at 210 nm.

A second gradient profile was evaluated by injecting 2 µL of a sample containing 100 µg/mL creatinine, caffeine and MPPA dissolved in pure water. The analysis method (Method B) was extended to 28 min with a flow rate of 0.500 mL/min and started with 99% ACN and 1% ammonium acetate, after which the percentage of ammonium acetate was increased over time as follows: isocratic, 0-2 min; 1-50% ammonium acetate, 2-20 min; isocratic, 20-25 min; 50-1% ammonium acetate, 25-27 min; isocratic, 27-28 min. A 5 min post-run ensured the system was at initial conditions. Absorbance was measured at 214 nm.

4.3.3.3 Modified methods for the separation of metabolites

Chromatographic conditions were the same as for establishing metabolite R_t -values (subsection 4.3.3.2). These conditions were kept constant with respect to the column, injection volume, absorbance detection wavelength and post-run time. Adjustments were only made to the solvent system, analysis time and gradient profiles for the various modified methods with respect to Method B as described in the previous subsection.

Method C

The binary solvent system of the analysis method was changed to consist of 10 mM ammonium formate (pH 4.48) in water and ACN, respectively. The same gradient profile as Method B was used.

Method D

The binary solvent system of the analysis method was changed to consist of 0.05 % (v/v) formic acid (pH 4.5) in water and ACN, respectively. The same gradient profile as Method B and C was used.

Method E

The analysis time of the method was adjusted to 24 min and gradient profile changed to start with 99% ACN and 1% formic acid, after which the percentage of formic acid was increased over time as follows: isocratic, 0-2 min; 1-55% formic acid, 2-20 min; isocratic, 20-22 min; 55-1% formic acid, 22-23 min; isocratic, 23-24 min. The rest of the conditions remained unchanged with respect to Method D.

4.3.4 Procedures for establishing calibration curves

Stock solutions containing 1 mg/mL standard were prepared for creatinine, caffeine and MPPA in distilled water. Dilutions of each standard stock were made with distilled water. Two detectors were used, namely the variable wavelength detector (VWD) and the mass spectrometer detector (MSD) resulting in four sets of calibration curves: a calibration curve each for creatinine and caffeine with the different detectors. The sets contained either creatinine or caffeine as the analyte and MPPA as internal standard in each case. The standard mixtures were prepared in triplicate to contain 5, 10, 25, 50, 75 and 100 $\mu\text{g/mL}$ of metabolite and 50 $\mu\text{g/mL}$ of the internal standard for analysis of samples in the same concentration range.

Each set of standards was analyzed in triplicate by LC–UV–MS with Method E and blank runs were performed after every sample run to ensure that there was no carry-over between runs. The peak areas of interest were obtained by manual integration and calibration curves of peak area versus concentration were constructed for both the LC–UV and MS extracted ion chromatograms. The MS extracted ion chromatograms were obtained for ions at m/z of 114, 150 and 195 for creatinine, MPPA and caffeine, respectively. Calibration curve sets were fitted with linear equations over the concentration range of 5–100 µg/mL by linear regression in SigmaPlot® 11.0.

$$y = mx + c \quad \text{Eq. 9}$$

4.3.5 Preparation of unknown and urine sample

The three samples with unknown amounts of creatinine and caffeine were prepared independently by a laboratory at the Department of Biochemistry, Stellenbosch University (Stellenbosch, South Africa). Urine from a subject was collected over 24 hours. After each collection, the urine was immediately stored at 4°C. After 24 hours the collections were pooled and a sample was taken. Before analysis, the unknown and urine samples were kept at room temperature for 30 min and 1 mL was then diluted two times using a stock solution of 100 µg/mL of the internal standard, MPPA, in distilled water. The samples were then ready for LC–UV–MS analysis.

4.4 Results and discussion

4.4.1 Detection of metabolites by UV

Detection of creatinine, caffeine and MPPA by UV was evaluated to establish the optimum wavelength at which all three analytes absorb. Detection at 210, 214, 220, 230, 250 and 254 nm were considered with absorbance at 214 nm determined as optimum for all three analytes.

4.4.2 Separation of metabolites by HPLC

The development of an LC–UV–MS method required that appropriate stationary and mobile phases be selected. Thereafter, the next challenge was to optimize the solvent gradient for efficient separation of both metabolites in such a manner that baseline resolution and symmetric peak

shapes could be achieved. Only after both these objectives were achieved could the LC–UV–MS method be deemed suitable for quantitative studies. The details of the method development are discussed in the following subsections.

4.4.2.1 Reversed-phase HPLC method

We decided to investigate the use of a hydrophobic stationary phase based on the motivation provided in subsection 4.2.1, and to combine it with a polar mobile phase. A ZORBAX® SB-C18 was evaluated for separation of creatinine, caffeine and MPPA.

The ZORBAX® SB-C18 column contains sterically-protected hydrophobic C18 chains chemically bonded to high purity ZORBAX® porous silica microspheres. The column is especially suited in applications that utilize high-sensitivity detectors that require low backgrounds, e.g., mass spectrometers. The column was evaluated using a binary solvent system consisting of ammonium acetate and ACN to elute the analytes from the column. The predicted elution order of the analytes (creatinine → caffeine → MPPA) could not be confirmed due to creatinine and caffeine being the only metabolites to elute from the column using the isocratic method. The internal standard, MPPA, was strongly retained on the column and could only be eluted with a gradient-based method containing more than 75% ACN. Sufficient retention of creatinine could not be achieved with an isocratic or gradient-based solvent system, therefore this method was not explored further.

4.4.2.2 HILIC-based separation method

The BETASIL® Diol-100 column was evaluated using a mobile phase consisting of ammonium acetate and ACN. An isocratic-based solvent system proved to be insufficient to confirm the predicted elution order of the analytes in HILIC (MPPA → caffeine → creatinine) since both creatinine and MPPA did not elute from the column under these conditions. A gradient elution system (Method A) was found to give sufficient separation of the analytes to allow for development of an HPLC-based method.

The R_t of the analytes was determined from the resulting HPLC chromatogram and are 5.428, 8.748 and 9.362 min, respectively. These values did not confirm the elution order, namely MPPA → caffeine → creatinine, predicted by the $c \log P$ values. This is most likely due to the fact that creatinine and MPPA are in a charged state under the mobile phase conditions and the $c \log P$ values

are only applicable to neutral compounds. $\log D$ values calculated based on the pK_a values of the analytes predict the elution order to be caffeine \rightarrow MPPA \rightarrow creatinine, which is indeed observed, although the elution of MPPA was expected to be closer to caffeine than to creatinine.

Subsequent HPLC analyses of different analyte concentrations revealed elevated background interferences. The detection wavelength was adjusted to 214 nm in an attempt to lower the background absorption. Furthermore, the analysis time increased (Figure 4.1) and the flow rate halved to operate closer to the optimal flow rate in HILIC. Although some of the initial background absorption was found to decrease (Figure 4.2.), this could present a problem for future application of the method to separate and quantify urinary metabolites.

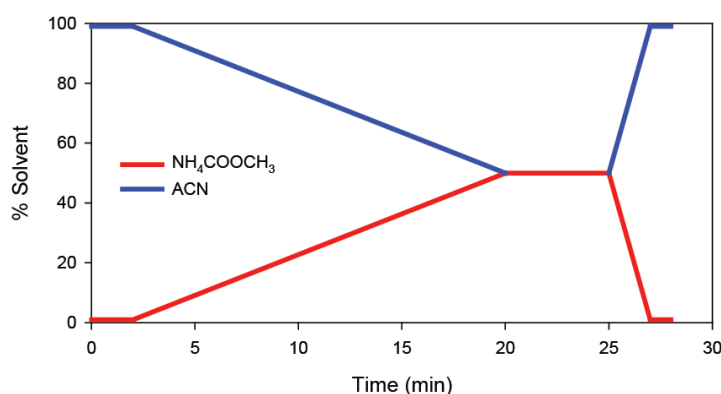


Figure 4.1. Gradient profile (Method B) used for the elution of creatinine, caffeine and MPPA. Modifications were made to analysis time (extended from 12 to 28 min), flow rate (changed from 1.000 to 0.500 mL/min) and detection wavelength (changed from 210 to 214 nm) compared to Method A.

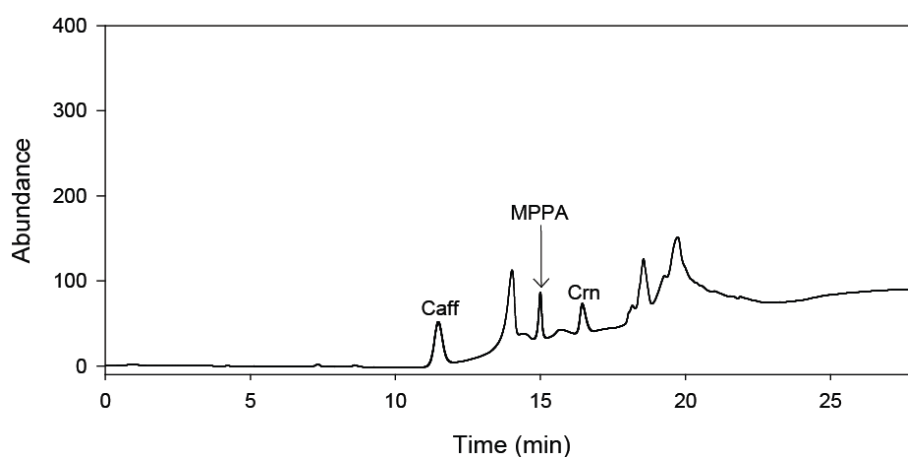


Figure 4.2. Analysis of 100 $\mu\text{g/mL}$ creatinine, caffeine and MPPA by HILIC using Method B with detection at 214 nm. The retention times of creatinine, caffeine and MPPA are 16.452, 11.478 and 14.997 min respectively.

Ammonium acetate and ammonium formate both absorb at 214 nm [123] which was observed in the current method for the former. The solvent system was therefore deemed unsuited for further investigation and needed to be modified because of the presence of high background absorption.

4.4.2.3 Modified gradient-based separation methods

In the first prominent modification (Method C, Figure 4.3), the solvent system was changed to 10 mM ammonium formate and ACN to compare the background absorption to that of the previous solvent system. All the other conditions remained unchanged. A reduction in the number of blank peaks was observed, with only the peaks of the analytes present (Figure 4.4). Caffeine eluted as a relatively broad peak and the separation of creatinine and MPPA was not adequate. Subsequent modifications of Method C included a combination of extended analysis time and modified gradient profiles which proved insufficient in resolving these issues.

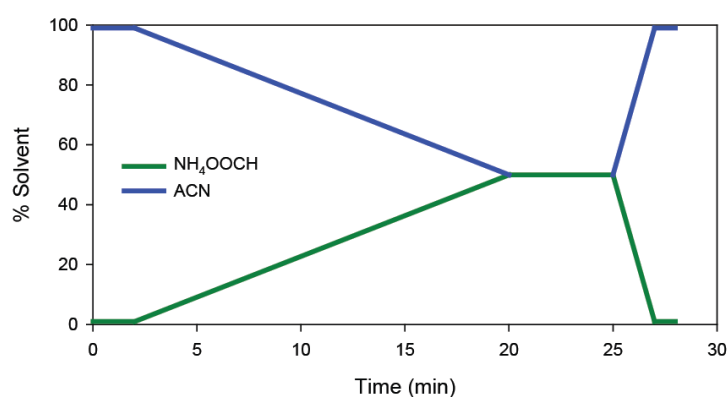


Figure 4.3. Gradient profile for Method C used for the separation of creatinine, caffeine and MPPA. The solvent system was modified to consist of ammonium formate and ACN.

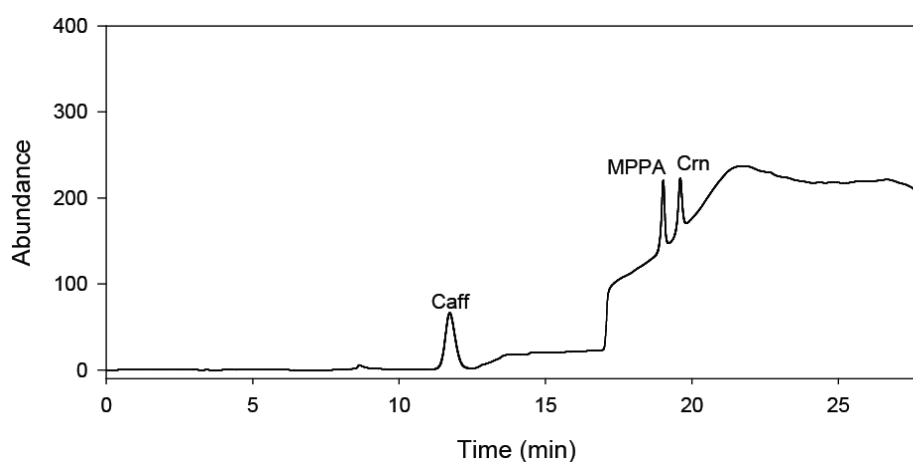


Figure 4.4. Analysis of 100 µg/mL creatinine, caffeine and MPPA with ammonium formate/ACN. The retention times of creatinine, caffeine and MPPA are 19.600, 11.728 and 19.020 min respectively.

In the second prominent modification (Method D), the solvent system was changed to 0.05 % (v/v) formic acid in water and ACN to compare the resulting resolution and peak shapes to those of the previous solvent system. All the other conditions were the same as for Method C. The peak shape of caffeine was enhanced, although it needed further optimization. The separation of creatinine and MPPA was improved, although it was still inadequate.

The third prominent modification (Method E) entailed the optimization of Method D. Subsequent analyses with modified gradient profiles revealed that increased separation of the creatinine and MPPA peaks could be achieved with a solvent system consisting of 1% of a 0.05% (v/v) formic acid in water and 99% ACN at the start, which was varied according to the following gradient: isocratic, 0-2 min; 1-55% formic acid, 2-20 min; isocratic, 20-25 min; 55-1% formic acid, 25-27 min; isocratic, 27-28 min (Figure 4.5). The resulting separation is shown in Figure 4.6.

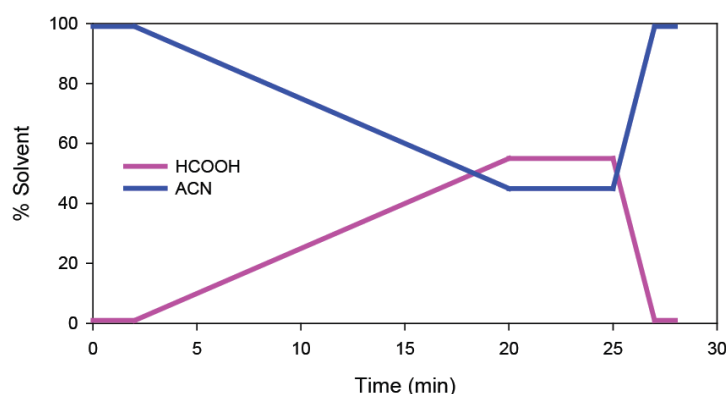


Figure 4.5. Gradient profile of Method E for the separation of creatinine, caffeine and MPPA. The solvent system was modified to consist of 0.05% (v/v) formic acid in water and ACN, and the gradient profile was adjusted.

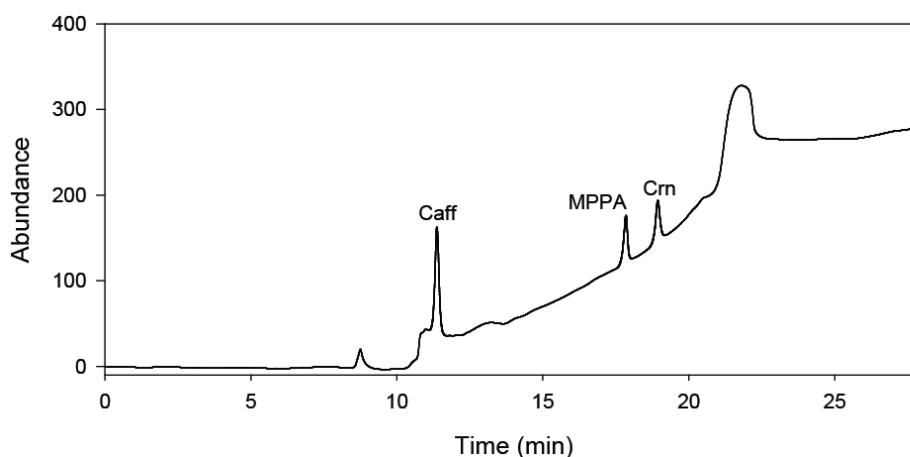


Figure 4.6. Analysis of 100 µg/mL creatinine, caffeine and MPPA with formic acid/ACN. The retention times of creatinine, caffeine and MPPA are 17.938, 11.315 and 16.738 min respectively.

Closer inspection of the resulting chromatogram confirms that the separation of the analytes occurs within the first 20 min of Method E. The analysis time was changed to 24 min in order to optimize the time and decrease the amount of solvent consumed during the analysis (Figure 4.7). This simplified and shortened method proved to be the most successful gradient method for the separation of the analytes using HILIC. Using this method, analyte standards gave R_t values shown in Table 4.2.

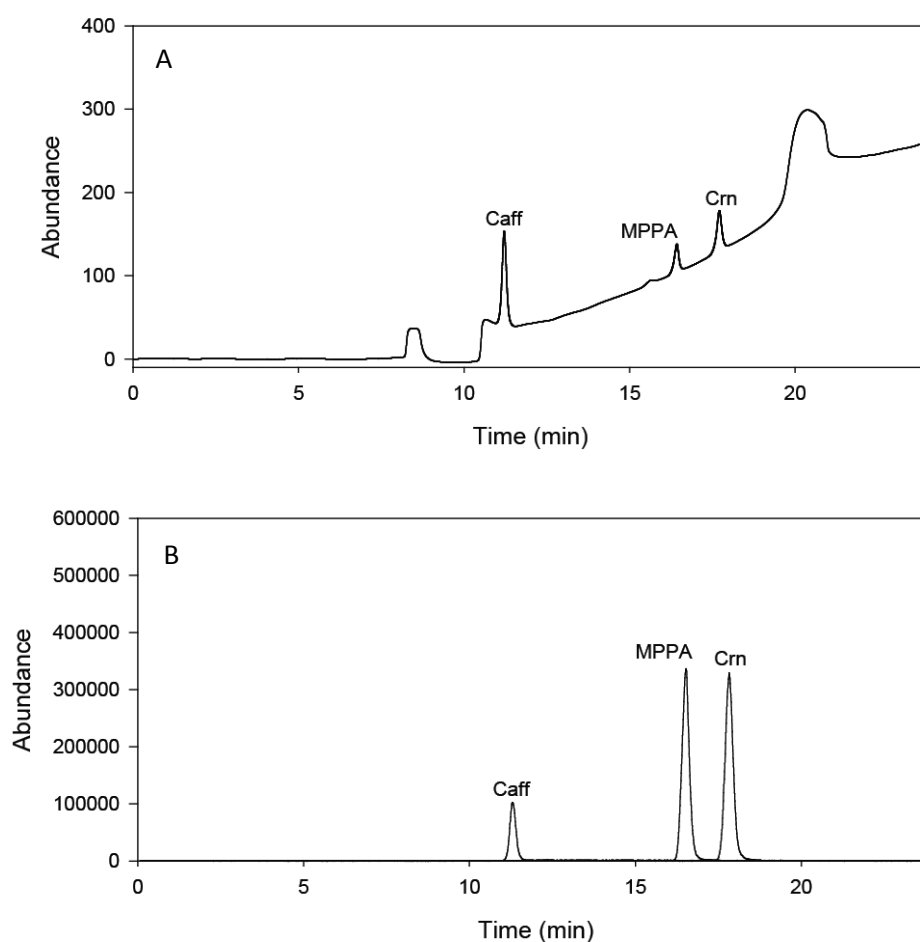


Figure 4.7. Analysis of 75 µg/mL creatinine, caffeine and MPPA using the optimized HILIC method. Chromatogram A: UV detection at 214 nm. Chromatogram B: Overlay of extracted ion chromatograms for creatinine (m/z 114), caffeine (m/z 195) and MPPA (m/z 150). The retention times of creatinine, caffeine and MPPA are 17.713, 11.213 and 16.428 min respectively.

Table 4.2. Retention times of urinary metabolites and internal standard, MPPA, with gradient-based HPLC Method E.

Standard	R_t (min)
Caffeine	11.213
MPPA	16.428
Creatinine	17.713

4.4.3 Metabolite quantification

The internal standard method was employed for the quantification of the urine metabolites: creatinine and caffeine. Quantification was achieved by generating calibration curves for the metabolites as described in the following subsection.

4.4.3.1 Calibration curves

Standards of creatinine and caffeine were prepared and 50 µg/mL of the internal standard, MPPA, was added to the respective sets of standards. The creatinine standards covered a range of concentrations similar to those expected to be found in urine, taking into account a 10-fold dilution that occurs during sample preparation. Determination of the resulting response using both UV (214 nm) and MS (extracted ion chromatogram) signals allowed calibration curves for both metabolites to be obtained over a final concentration range of 5–100 µg/mL (Figure 4.8).

The LC–UV calibration curves displayed a linear relationship over the concentration range of 5–100 µg/mL for both analytes. The LC–MS extracted ion calibration curves displayed a linear relationship over the concentration range of 5–25 µg/mL for both analytes, with large discrepancies at higher analyte concentrations. From the calibration curves, it is evident that quantification by LC–UV is more reliable than using LC–MS. Linearity is affected to a greater extent at higher analyte concentrations in MS detection possibly due to space charge effects at these levels.

4.4.3.2 Limits of detection and quantification

The ability of the developed method to accurately detect and quantify the urine metabolites in artificial samples was subsequently assessed in terms of the limit of detection (LOD) and limit of quantification (LOQ). The LOD of a specific analyte is defined as the lowest concentration of the analyte that can be detected that is statistically different from an analytical blank. The definition of LOQ is the lowest concentration of a specific analyte which can be quantitatively analyzed with reasonable reliability [132].

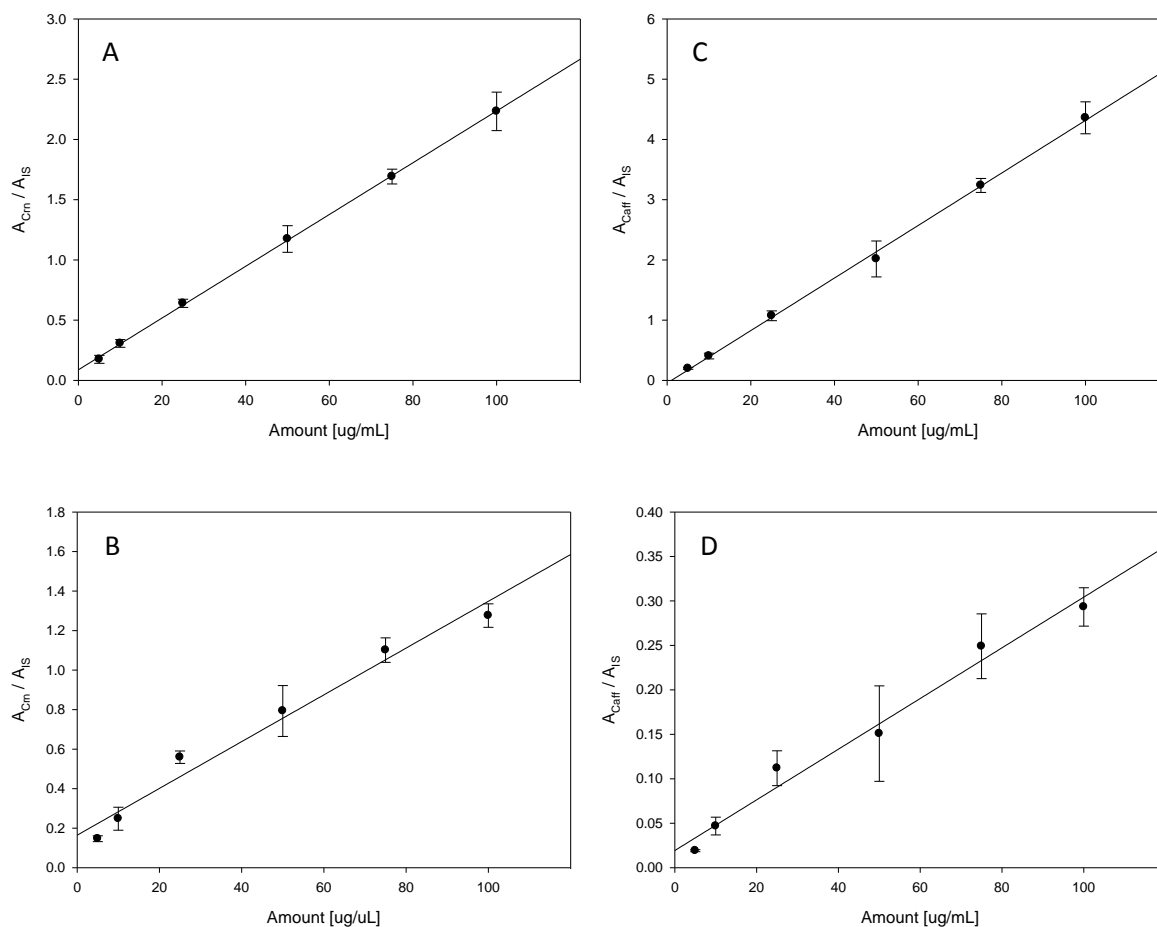


Figure 4.8. Calibration curves of the urinary metabolites. A and B) Creatinine calibration curves; C and D) Caffeine calibration curves. A and C were constructed using UV detection at 214 nm. B and D were constructed using MS extracted ion chromatograms (m/z 195 and 114 for caffeine and creatinine, respectively). Linear regression of A, B, C and D resulted in R^2 values of 0.991, 0.957, 0.990 and 0.927 respectively. All data points represent the mean of triplicate values, with the error bars denoting the standard deviation.

External calibration curves (not shown here) were constructed for the determinations of the LODs and LOQs, which are calculated by considering σ , the residual standard deviation of the regression line (also known as the standard error of estimate of regression). Calculating σ in this manner gives a value that is expressed in the same units as that used for detection, i.e. the same units as the y-axes of the calibration curves. By substituting σ for y in the linear calibration equation ($y = mx + c$), σ can also be expressed in terms of concentration. Based on a 95% confidence level, the LOD is normally considered to be equal to 3σ and LOQ as equal to 10σ [132], although in this case the LOD and LOQ for all the urinary metabolites of interest need to be calculated from the external calibration curves using equations 10 and 11 respectively,

$$LOD(\mu\text{g} / \text{mL}) = \frac{3\sigma - y_0}{m} \quad \text{Eq. 10}$$

$$LOQ(\mu\text{g} / \text{mL}) = \frac{10\sigma - y_0}{m} \quad \text{Eq. 11}$$

where y_0 is the y-intercept and m is the slope of the (external standard) linear calibration regression line. The results are shown in Table 4.3.

Table 4.3: LOD and LOQ values for the urinary metabolites of interest obtained using LC–UV–MS Method E.

Compound	Detection method	σ	Slope	Intercept	R^2	LOD $\mu\text{g}/\text{mL}$	LOQ $\mu\text{g}/\text{mL}$	Analysis range $\mu\text{g}/\text{mL}$
Creatinine	UV	27.753	5.739	29.492	0.983	9.369	43.222	5 – 100
	MS	479103	67135	913082	0.963	7.809	57.764	5 – 100
Caffeine	UV	49.742	11.471	8.259	0.986	12.288	42.642	5 – 100
	MS	116031	15219	121037	0.958	14.919	68.286	5 – 100

LOD and LOQ are very strict measurements of accuracy and an analytical method that gives very low variability in the obtained values is required to produce satisfactory values. Lower LODs are expected for detection of both creatinine and caffeine by MS than by UV. However, the calculated values indicate a lower LOD for the detection of only creatinine by MS. This is partially due to the larger variability in the MS data for caffeine, especially at higher concentrations, which results in higher LOD and LOQ values calculated according to the method described above (even though the extracted ion chromatogram signals at these levels show good intensity). The linearity of the calibration curves indicate high variability in the standard deviations of caffeine and therefore affect the reproducibility of this detection method. Selected ion monitoring (SIM) can potentially address this problem, but was not explored in the current study. Furthermore, ionization of caffeine is likely not favored under the mobile phase conditions used here.

The LOQ values for both creatinine and caffeine by UV detection are lower compared to the values obtained by MS detection. Despite UV being less selective and sensitive at low wavelengths, it is better suited for the quantification of the target analytes than MS under the LC conditions used here. It is important to point out though that using UV detection, compound identification is based solely on R_t 's of the compounds of interest, which is not very reliable at low wavelengths. MS in scan mode on the other hand allows identification of the compounds of interest by their mass spectra.

The LC–UV–MS method developed here therefore combines the two detection methods to complement one another in order to perform both qualitative (MS) and quantitative (UV) analysis.

4.5 Quantification of unknown samples

The ability of the developed method to accurately detect and quantify creatinine and caffeine as urinary metabolites was evaluated.

The internal standard, MPPA, was added to three artificial samples, prepared independently, containing unknown amounts of creatinine and caffeine, and analyzed using the developed LC–UV–MS method. The concentrations and recoveries were successfully calculated for different concentrations in each sample using the LLS equations of the respective calibration curves in Figure 4.10. The results are reported in Table 4.4.

Table 4.4. Quantitative results for samples with unknown amounts of creatinine and caffeine. All the data represent the mean of triplicate values, with the standard deviation noted.

Compound	Sample	Theoretical concentration µg/mL	LC–UV*		LC–MS*	
			Measured concentration µg/mL	% recovery	Measured concentration µg/mL	% recovery
Creatinine	<i>Unknown 1</i>	67.5	66.8±1.7	99.0±2.5	68.4±0.9	101±1.3
	<i>Unknown 2</i>	22.5	22.4±0.4	99.4±1.8	23.8±0.3	106±1.1
	<i>Unknown 3</i>	15.0	15.0±0.2	100±1.0	15.1±0.6	101±3.7
Caffeine	<i>Unknown 1</i>	85.0	87.8±1.2	103±1.5	83.5±6.3	98.3±7.4
	<i>Unknown 2</i>	12.5	13.0±0.4	104±3.0	10.7±2.8	85.3±22.6
	<i>Unknown 3</i>	60.0	61.7±1.7	103±2.9	72.1±1.5	120±2.5

* Using HILIC Method E and the calibration data reported in Figure 4.8.

4.6 Comparison of results

4.6.1 *F*-test and Student's *t*-test

In the comparison of the values obtained from a set of results with either the true value or other sets of data, it is possible to determine whether an analytical procedure provides accurate and/or precise values, or if one method is superior to another. The two most common methods for comparing results are the *F*-test (the variance ratio test) and the Student's *t*-test [132].

When a new analytical method is being developed it is common practice to compare the values for the mean and precision of the new test method with those of an established (reference) procedure. In this case the comparison was done between three newly developed analytical methods: DI-ESI-MS, LC-UV and LC-MS.

The *F*-test is used to compare the precision of two sets of data obtained from the results of two different analytical methods. The *F*-value calculated from the equation:

$$F = \frac{s_A^2}{s_B^2} \quad \text{Eq. 12}$$

where s_A and s_B are the standard deviations for data set A and B, respectively.

There must not be a significant difference between the precision of the methods. Hence the *F*-test is applied prior to using the Student's *t*-test.

The Student's *t*-test is used for small sample numbers and its purpose is to compare the mean from a sample with some standard value and to express some level of confidence of the comparison. The value of *t* when comparing two sample means \bar{x}_1 and \bar{x}_2 is given by the expression:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_p \sqrt{1/n_1 + 1/n_2}} \quad \text{Eq. 13}$$

where n_1 and n_2 are the number of observations for two samples, respectively, and s_p is the pooled standard deviation, calculated from the two sample standard deviations s_1 and s_2 , as follows:

$$s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}} \quad \text{Eq. 14}$$

The F -test was employed to compare the precision of the results of the DI–ESI–MS method with the LC–UV–MS method using both UV and MS data, and also comparing the UV and MS data with each other. The mean values obtained using these three methods for the three samples with unknown amounts of creatinine and caffeine were used for this purpose. The value obtained for F was checked for its significance against values in the F -table calculated from an F -distribution corresponding to the degrees of freedom ($n - 1$, where $n = 3$) for the two sets of data. The F -values obtained for the three sets of data are tabulated in Table 4.5.

There is no significant difference at the 10 percent probability level between the precisions of five of the six sets of results when comparing data for the DI–ESI–MS method with those obtained by LC–UV; one set of results (creatinine in Unknown 3) clearly showed a significant difference. A comparison of the DI–ESI–MS method with the LC–MS method showed no significant difference at the 10 percent level for two of the three sets of results for caffeine, with the other set of results (caffeine in Unknown 1) showing a significant difference at the 1 percent level. The three sets of results for creatinine showed no significant difference at the 5 percent level between the values.

Three of the six sets of results in a comparison between the two methods of detection (UV and MS) used in combination with LC separation showed no significant difference at the 10 percent level, and one set of results at the 5 percent level. The remaining two sets of results (caffeine in Unknowns 1 and 2) reveal a significant difference between the two methods. The amount of caffeine in Unknown 1 and creatinine in Unknown 3 are close to the LODs for the respective compounds. Therefore the significant difference in the F -test comparisons for these two samples is expected.

Despite the significant differences between four of the sets of results, data for all three methods can be viewed as comparable due to no significant difference at the 5 percent level for most of the comparisons between the sets of results.

Table 4.5. *F*-test comparison of precisions of three different analytical methods. For $P = 0.10$, 0.05 and 0.01, the *F*-distribution for two degrees of freedom are 9.00, 19.00 and 99.00, respectively.

Compound	Sample	<i>F</i> -test comparisons		
		DI-ESI-MS vs.	DI-ESI-MS vs.	LC-UV vs.
		LC-UV	LC-MS	LC-MS
Creatinine	Unknown 1	3.23 ^{a,b}	12.49 ^b	3.87 ^{a,b}
	Unknown 2	4.84 ^{a,b}	12.47 ^b	2.58 ^{a,b}
	Unknown 3	217.29 ^c	16.35 ^b	13.29 ^b
Caffeine	Unknown 1	1.29 ^{a,b}	19.69 ^c	25.41 ^c
	Unknown 2	7.81 ^{a,b}	7.15 ^{a,b}	55.84 ^c
	Unknown 3	5.08 ^{a,b}	3.72 ^{a,b}	1.37 ^{a,b}

^a *F*-value indicates no significant difference between the precision of the two sets of results at the 10 percent level.

^b *F*-value indicates no significant difference between the precision of the two sets of results at the 5 percent level.

^c *F*-value indicates a significant difference between the precision of the two sets of results at the 1 percent level.

The exceptions in all cases are where the analyte of interest is close to the LOD value of a particular method. The Student's *t*-test can therefore be used with confidence and the values are shown in Table 4.6.

Table 4.6. Student's *t*-test values for the comparison of the three analytical methods. For $P = 0.10$, 0.05 and 0.01, the *t*-distribution for two degrees of freedom are 2.92, 4.30 and 9.93, respectively.

Compound	Sample	<i>t</i> -test comparisons		
		DI-ESI-MS vs.	DI-ESI-MS vs.	LC-UV vs.
		LC-UV	LC-MS	LC-MS
Creatinine	Unknown 1	0.72 ^a	0.02 ^a	1.55 ^a
	Unknown 2	0.05 ^a	2.12 ^a	4.62 ^c
	Unknown 3	0.19 ^a	0.12 ^a	0.06 ^a
Caffeine	Unknown 1	3.59 ^b	0.24 ^a	0.99 ^a
	Unknown 2	0.88 ^a	0.86 ^a	1.14 ^a
	Unknown 3	2.25 ^a	11.75 ^d	9.43 ^c

^a *t*-value indicates no significant difference between the mean results of the two methods at the 10 percent level.

^b *t*-value indicates no significant difference between the mean results of the two methods at the 5 percent level.

^c *t*-value indicates a significant difference between the mean results of the two methods at the 1 percent level.

^d *t*-value indicates a highly significant difference between the mean results of the two methods below the 1 percent level.

There is no significant difference at the 10 percent probability level between the precision of five of the six sets of results (and one set of results at the 5 percent level) when comparing the DI-ESI-MS method with the LC-UV method. Data for five of the six sets of results obtained by the DI-ESI-MS and the LC-MS method showed no significant difference at the 10 percent level and the remaining set of results (creatinine in Unknown 3) reveals a significant difference between the two methods. A comparison of the LC-UV method with the LC-MS method showed no significant difference at the 10 percent level for four of the six sets of results with two sets of results (creatinine in Unknown 2 and caffeine in Unknown 3) having a significant difference at the 1 percent level.

4.6.2 Paired *t*-test

The paired *t*-test was also employed to validate the DI-ESI-MS method with the LC-UV and LC-MS methods, and the last two methods with each other. The three methods were used for the analysis of the three samples with differing unknown amounts of creatinine and caffeine, with the results of only the DI-ESI-MS and the LC-UV methods reported in Table 4.7 as an example.

Table 4.7. Calculation of the differences between the results of the DI-ESI-MS and the LC-UV methods. The differences between each pair of results (d) and the mean of the difference (\bar{d}) is obtained. \bar{d} is subtracted from d for each pair of results and the square of each value is calculated. The sum of all the squares ($\sum (d - \bar{d})^2$) is used in the calculation of the standard deviation in order to determine the *t*-distribution value.

Compound	DI-ESI-MS	LC-UV	d	$d - \bar{d}$	$(d - \bar{d})^2$
Creatinine	68.4	66.8	1.5	0.9	0.83
	22.4	22.4	0.0	-0.6	0.35
	15.3	15.0	0.3	-0.3	0.10
			$\sum d = 1.9$	$\sum (d - \bar{d})^2 = 1.28$	
			$\bar{d} = 0.6$		
Caffeine	82.5	87.8	-5.3	-2.9	8.54
	12.3	13.0	-0.7	1.7	2.93
	60.5	61.7	-1.2	1.2	1.47
			$\sum d = -7.1$	$\sum (d - \bar{d})^2 = 12.98$	
			$\bar{d} = -2.4$		

The differences (d) between each pair of results are calculated and \bar{d} , the mean of the difference, is obtained. \bar{d} is subtracted from d for each pair of results and the square of each value is

calculated. The sum of all the squares ($\sum (d - \bar{d})^2$) is used in the calculation as showed in equation 15. The standard deviation (s_d) of the differences is evaluated and the t -distribution is calculated using equations 15 and 16.

$$s_d = \sqrt{\frac{\sum (d - \bar{d})^2}{n-1}} \quad \text{Eq. 15}$$

$$t = \frac{\bar{d} \sqrt{n}}{s_d} \quad \text{Eq. 16}$$

Data obtained with the DI-ESI-MS and LC-UV methods were also compared with those obtained by LC-MS. The calculated t -distribution values of the three method comparisons are reported in Table 4.8.

Table 4.8. Paired t -test comparison of three different analytical methods. For $P = 0.10$, 0.05 and 0.01 , the t -distribution for two degrees of freedom are 2.92, 4.30 and 9.93, respectively.

Compound	t-distribution		
	DI-ESI-MS vs.	DI-ESI-MS vs.	LC-UV vs.
	LC-UV	LC-MS	LC-MS
Creatinine	1.35	0.79	2.13
Caffeine	1.62	0.91	0.28

There is no significant difference at the 10 percent level between the quantitative data obtained by any of the three methods.

4.7 Quantification of urine sample

After successfully using the LC-UV-MS method to quantify artificial samples, the method was evaluated for the analysis of a urine sample to detect creatinine and caffeine. The internal standard, MPPA, was added to a urine sample diluted two-fold and analyzed using Method E to determine the sensitivity of the method. The resulting LC-UV and extracted ion chromatogram as well as the mass spectra for creatinine, caffeine and MPPA of the analyzed urine sample are shown in Figure 4.9.

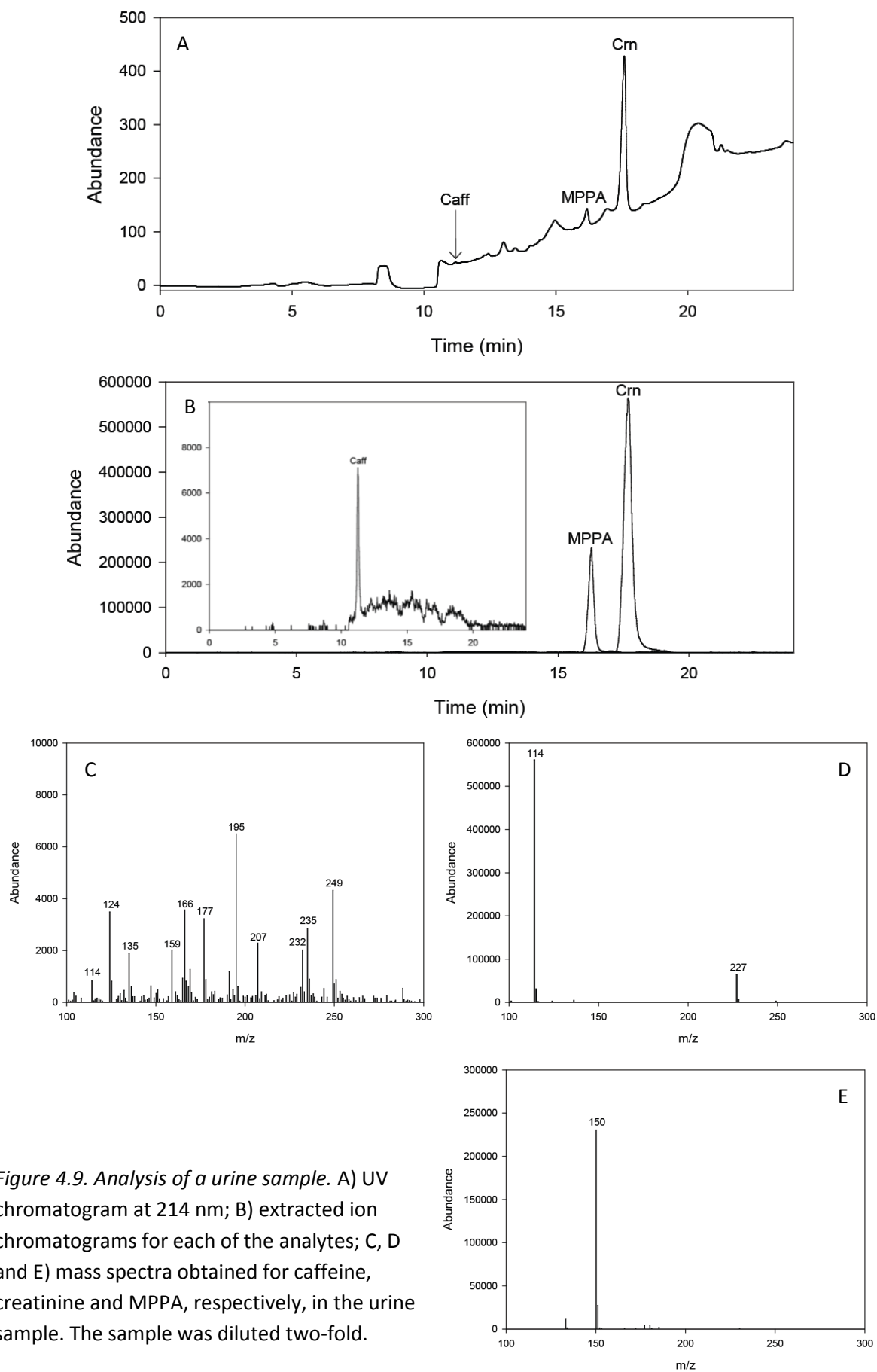


Figure 4.9. Analysis of a urine sample. A) UV chromatogram at 214 nm; B) extracted ion chromatograms for each of the analytes; C, D and E) mass spectra obtained for caffeine, creatinine and MPPA, respectively, in the urine sample. The sample was diluted two-fold.

The mass spectra for each peak in the extracted ion chromatogram confirmed the presence of the respective compounds. The signal for caffeine (195 m/z) was very low and many ions due to the mobile phase and matrix are observed as shown in Figure 4.9 C. In mass spectrum D both creatinine (114 m/z) and its dimer (227 m/z) are present; with mass spectrum E confirming the presence of MPPA (150 m/z). In both the LC–UV and LC–MS analyses, caffeine was close to the LOD rendering its quantification impossible. The sample needed to be diluted ten-fold to ensure creatinine was within the calibration range, but was not performed since the detection of caffeine would not be possible at such a high dilution factor. Therefore we were unable to perform simultaneous quantification for both compounds in a urine sample.

Chapter 5: Conclusion

5.1 Overview of achievements

The use of creatinine as internal standard presents a valuable tool in clinical and forensic studies. This thesis presents an initial study on the detection and quantification of creatinine, creatine and caffeine by direct infusion mass spectrometry. The reason for this research is to overcome adulteration problems such as the deliberate addition of water to dilute urine samples by using creatinine as an endogenous control substance against which concentration levels of metabolite of interest can be compared. The study addressed this problem by development of a direct infusion mass spectrometry analytical method for detection and quantification of creatinine, creatine and caffeine.

In pursuit of this aim, a simple and sensitive analysis method was developed based on a direct infusion of the metabolites into a mass spectrometer. Key parameters for the direct infusion were established by considering the composition of the analyte solution, ionization of the compounds, sample introduction, separation and detection of ions, and the use of a suitable internal standard for quantitative purposes. The analyte solution contained methanol, water and acetic acid. Methanol ensured low viscosity and volatility of the carrier solvent whilst water was included due to the metabolites being highly polar. Acetic acid promoted quantitative ionization in positive ionization mode. Sample introduction was performed by continuous infusion of the analyte solution and the solution entered the spray needle by gravitational force alone. Solvent pumping was not required. This ensured consistent and reproducible flow speed. To ensure the analyte molecules were in monomeric form and did not form clusters the fragmentation voltage was set at 95 V. 1-Methyl-3-phenylpropylamine was selected as internal standard since it met all the requirements to determine the concentration of creatinine. Establishment of these conditions rendered the use of the direct infusion electrospray ionization mass spectrometry (DI-ESI-MS) method possible.

A positive highlight of the DI-ESI-MS method was the successful construction of calibration curves for creatinine, creatine and caffeine with good linearity over a concentration range of 1–10 µg/mL. The linear regression of the calibration curves resulted in R^2 values of 0.985, 0.997 and 0.977 for

creatinine, creatine and caffeine, respectively. Limit of detection (LOD) of 0.597 µg/mL, 0.073 µg/mL and 1.427 µg/mL was obtained for creatinine, creatine and caffeine, respectively. Applicability of this method was confirmed by successfully quantifying three artificial samples containing unknown amounts of compounds of interest with mean recoveries of 99.6–102% for creatinine and 97.1–101% for caffeine. The DI–ESI–MS method requires minimal sample pretreatment rendering it fast and facile with a total analysis time of 5 min. It was found that the calibration curve for caffeine could not be used in the application of the method in analysis of a human urine sample due to the low concentration of caffeine present compared to the concentration of creatinine.

A liquid chromatography (LC) method with ultraviolet (UV) and mass spectrometry (MS) detection was developed using a BETASIL® Diol-100 HILIC column to validate the DI–ESI–MS method. A successful elution protocol was developed and optimized for the separation of creatinine, caffeine and the internal standard, MPPA. The chromatographic analysis procedure was subsequently employed to obtain linear calibration curves over a concentration range of 5–100 µg/mL for creatinine and caffeine quantification using the two detectors, UV and MS. The LODs for creatinine with UV and MS were 9.369 and 7.809 µg/mL, respectively. Caffeine had LODs of 12.288 µg/mL and 14.919 µg/mL with UV and MS, respectively. These LODs are significantly higher than those reported for the DI–ESI–MS method (in part due to the poor reproducibility of manual injection). The analysis of three artificial samples containing unknown amounts of compounds of interest were performed with mean recoveries of 99.0–100% for creatinine and 103–104% for caffeine when using LC–UV. Analysis using LC–MS resulted in mean recoveries of 101–106% for creatinine and 85.3–120% for caffeine. These values are comparable to the percentage recoveries obtained with the DI–ESI–MS method.

Statistical analysis, including the *F*-test, Student's *t*-test and paired *t*-test, of the data obtained by DI–ESI–MS, LC–UV and LC–MS methods proved that a significant difference between the methods at a confidence level of 95% does not exist, except for samples with amounts of compound close to the LOD. Therefore the DI–ESI–MS method was successfully validated by the LC–UV–MS method.

5.2 Future work

Problems encountered with the simultaneous quantification of creatinine and caffeine in a human urine sample by the DI–ESI–MS method that was developed in this work, are due to the low amounts of caffeine present rather than the analytical method itself. This is evident from the

relatively higher limits of detection for caffeine compared to creatinine and creatine. Construction of a calibration curve for creatine and caffeine can prove to be more useful since both compounds are present at lower concentrations in urine. Direct infusion mass spectrometry has potential for quantitative determinations due to its sensitivity, high selectivity, wide dynamic range, robustness, and the ability to identify metabolites. Furthermore it has the greatest potential for high sample throughput with minimal or no sample pretreatment. Introduction of samples via paper or fibers could be a possibility for direct analysis of samples and to improve electrospray ionization of the target molecules for quantitative purposes. Alternative mass analyzers can also be employed to greatly contribute to the sensitivity of direct infusion mass spectrometry. DI-ESI-MS can be applied in various chemical and biochemical fields, including clinical and forensic toxicology, anti-doping tests in sports performance studies, food analysis, and metabonomic and metabolomic studies.

The difficulties encountered with the LC-UV-MS validation method developed in this study can be attributed to the conversion of peak areas to metabolite concentrations. The relatively high limits of detection could in part be the result of poor injection reproducibility using manual injection. In order to accurately convert peak areas to concentrations, more reliable calibration curves will be required. In future these calibration curves will be constructed by analysis of standards of which the absolute concentrations has been confirmed by an independent method such as the classical Jaffé reaction.

Caffeine was selected as a model compound to represent common drugs or drug metabolites that may be analyzed. The analysis of real drugs or drug metabolites can in future performed using this DI-ESI-MS method.

5.3 Final remarks

The results presented in this work represent the successful development of the analytical method for the detection and quantification of creatinine and caffeine in artificial samples. This method allowed for fast, direct and facile determinations of the two compounds which hold significant potential for analyzing real drugs or drug metabolites present in human urine samples and pave the way for future direct infusion mass spectrometry studies.

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